REMARKS

Applicant's undersigned representative wishes to express his gratitude to Examiners Marvich and Jeffers for their courtesies during an office interview on November 10, 2004.

In the Advisory Action dated September 22, 2004, the Examiner indicated that the Supplemental Amendment filed August 26, 2004 was treated as a response to the Final Office Action but did not place the case in condition for allowance, and that the claim amendments therein were not entered. By virtue of the concurrently filed RCE, applicant has amended the claims and added new claims 86 and 87. It is respectfully submitted that, as is discussed in more detail below, all claim amendments are adequately supported by the application as originally filed and do not raise any issues of new matter. For example, support for the new claims can be found at Page 4, line 24 to page 5, line 4 and page 10, lines 14 to 19 of the Specification. Entry of the claim amendments and favorable reconsideration is respectfully requested.

Claim Rejections under 35 U.S.C. § 112, Second Paragraph

Applicant respectfully submits that the above claim amendments and cancellations have overcome or rendered moot all claim rejections under 35 U.S.C. § 112, ¶ 2. Specifically, with regard to the recitation of "viral-based vector," the claims have been amended to recite "viral vectors;" "derived from" has been changed to "obtained from" per the Examiner's kind suggestions; "gene or part thereof encoding a polypeptide having P450 activity" has been replaced or

deleted; claim 63 has been amended to recite that the promoter controls the expression of a polynucleotide; and claim 64 has been cancelled.

Claim Rejections under 35 U.S.C. § 112, First Paragraph

Applicant further submits that all rejections of the claims under 35 U.S.C. § 112, ¶ 1 have also been rendered moot or overcome by the claim amendments, and further in view of the information provided herein.

First of all, it is respectfully submitted that the "new matter" rejection and the "lack of written description" rejection due to the recitation of "genes encoding" "polypeptide having P450 activity" has been rendered moot by the amendments to the claims which no longer recite "genes" and now recite three specific P450 enzymes: CYP1A2, CYP2E1, and CYP3A4.

a. Written Description for CYP1A2, CYP2E1, and CYP3A4

During the Office Interview, the issue was raised that the recitation of CYP1A2, CYP2E1, and CYP3A4, and subsequent referral to CYP1A2, CYP2E1, and CYP3A4 of various origins indicate that each of them refers to a group of enzymes, and accordingly the specification might not contain sufficient written description for the three groups. This issue is not reflected in the written records. The following addresses this issue.

Cytochrome P450 (CYP) are widely distributed in nature, and are known to exist in almost all phyla, often with many forms appearing in a single organism. Many of them have been well-characterized. They have somewhat

conserved primary structure but vastly different substrates (about 20% sequence homology across organisms and across various forms, and a conserved core). See e.g. Graham & Peterson, 1999, Arch Biochem Biophys 369: 24–29 (Abstract attached as Exhibit 1).

Nevertheless, it has long been well-known that for CYP enzymes with similar functions, interspecific structural conservation is very high. For example, in a review article, Stoilov *et al.* (2001, Drug Metabol Drug Interact 18: 33–55, copy attached as Exhibit 2) stated that:

> Many of the forms of cytochrome P450 present in one species have homologues in other species. For example, CYP1A2 is present in many species, including man, rabbits, rodents, fish and fowl. The amino acid sequence identity of these homologues is often in excess of 70%. CYP26, too, has more than 61% identity in amino acid sequence between fish, fowl and mammals. In view of the high degree of conservation of sequence as well as of enzymatic activities, it is only reasonable to assume that such strong conservation of sequence also reflects a conservation of function.

Abstract of Stoilov *et al.* (2001) (*underline added*). Thus, an ordinarily skilled artisan would have recognized that the recitation "CYP1A2, CYP2E1, and CYP3A4" represents three group of enzymes which are well-known, well-characterized with highly conserved structural and functional characteristics. Many of the DNA sequences encoding these enzymes were available publicly in public databases, before the filing date of the instant application.

As added proof, applicant submits as Exhibit 3 sequence alignments of CYP1A2, CYP2E1, and CYP3A4 sequences from various organisms. They all

show that these sequences share over 70% sequence identify at the amino acid level. A summary of the comparison is shown in Table 1 below:

Table 1: Sequence Homology Among CYP1A2, CYP2E1, and CYP3A4 Enzymes From Various Organisms

CYP1A2		
Species comparison	**Sequence identity %**	**Sequence conservation* %**
Human to mouse	72	86
Human to guinea pig	76	87
Human to rat	74	87
CYP3A4		
Human to pig	75	86
CYP2E1		
Human to mouse	78	90
Human to rat	78	91
Human to dog	77	87
Human to pig	79	90
Human to hamster	78	91

**'Sequence conservation' includes both identical amino acids and conservative substitutions assumed to be functionally equivalent.*

Furthermore, as recognized by the Examiner in the Office Action, the specification teaches that "CYP1A2, CYP2E1, and CYP3A4 encode polypeptides that convert acetaminophen to NABQI which is a cytotoxic molecule." Accordingly, it is respectfully submitted that the specification, coupled with that which well-known to those skilled in the relevant art, provides adequate written description for the general of polynucleotides encoding CYP1A2, CYP2E1, and CYP3A4 enzymes.

In addition, it is well-known that acetaminophen has comparative toxicity across many mammalian species. See, for example, Entry for 4'-hydroxy-acetanilide (acetaminophen), Registry of Toxic Effects of Chemical Substances, maintained by the National Institute for Occupational Safety and Health

(available online at http://www.cdc.gov/niosh/rtecs/ae401640.html). This entry provides a summary of published toxicology for humans and other species. For acute toxicity, the intraperitoneal LD_{50} dose for mouse (367 mg/kg) and rat (1205 mg/kg) and the oral LD_{50} for guinea pig (2620 mg/kg) are provided. The lowest published lethal intravenous dose for pig (1000 mg/kg) and dog (826 mg/kg) are also listed. By comparison, the oral human acute toxicity data gives various lethal doses in the range 143 to 714 mg/kg. Thus, it is to be expected that the claimed GDEPT methods would be effective across many mammalian species, and can be effected with a suitable CYP1A2, CYP2E1, and CYP3A4 from many mammalian origins.

b. Enablement Related to Gene Therapy Technology

The Office Action continues to reject all claims for alleged lack of enablement, asserting that the claimed methods are complex which is further exacerbated by involving a gene therapy step. The reasoning of the Office Action in maintaining this rejection appears to be two-fold. First, the rejected claims recite a gene encoding a "P450 enzyme," but many of these genes are unknown, uncharacterized, or not known to have the desired function. Second, gene therapy is unpredictable and the specification exemplifies only with *in vitro* models.

It is respectfully submitted that the first basis of the claim rejection has been rendered moot because of the above claim amendments and discussion regarding the structural and functional conservancy of CYP1A2, CYP2E1, and

CYP3A4 enzymes across mammalian species. Furthermore, applicant hereby submits a Declaration, as Exhibit 4, which demonstrates that infection of cell lines derived from a range of tumor types with viral vectors carrying either human or mouse CYP1A2 genes sensitized these tumor cells to acetaminophen killing in a dose dependent manner.

With regard to the second aspect, the Declaration by the applicant shows that the claimed methods are effective with a mouse *in vivo* model. The data in the Declaration demonstrate that E1-deleted replication-defective adenoviruses expression human CYP1A2 and murine CYP1A2, when i.t. injected into mice with HepG2 xenografts, coupled with i.p. administration of acetaminophen, were effective in controlling tumor growth. The lack of *in vivo* data in the specification was mentioned in the Office Action numerous times and also during the Office Interview, and was the primary basis for the "lack of enablement" rejection. One reason articulated in the Office Action for the lack of enablement rejection is that the specification and the claims have not specified gene delivery doses and routes. The Office Action further cited several articles which theorize the possible difficulties related to gene therapy in general, such as difficulties in effective and targeted gene delivery. It is respectfully submitted that the Declaration, with its positive *in vivo* data, has clearly shown that these theoretical difficulties in gene delivery are not applicable to the method of the instantly claimed invention, and that no undue experimentation is required for one of ordinary skills in the art to determine effective doses and delivery routes for effective *in vivo* gene therapy.

The Declaration further demonstrated that modulation of cellular glutathione (GSH) levels using buthionine sulphoximine (BSO) improved cell killing efficacy; and that in mice, the mouse gene-based viral vector had improved cell-killing ability over the human gene-based vector, which is consistent with known species-specific acetaminophen toxicity.

In short, the data in the Declaration have demonstrated that, *in vivo*, a CYP1A2-mediated conversion of acetaminophen to NABQI is an effective and safe gene-directed enzyme prodrug therapy system for cancer treatment. Furthermore, human CYP1A2 was selectively inhibited with furafylline, the non-competitive hCYP1A2 inhibitor, suggesting that the liver of the patient can be further protected by selective inhibition of endogenous CYP1A2 activities. In other words, the claimed GDEPT methods are enabled and satisfy all requirements of 35 U.S.C. § 112.

In summary, applicant respectfully submits that all claims are in condition for allowance and earnestly solicit an early indication from the Examiner to that effect. If there are any questions regarding this amendment or the application in general, a telephone call to the undersigned would be appreciated since this should expedite the prosecution of the application for all concerned.

If necessary to effect a timely response, this paper should be considered as a petition for an Extension of Time sufficient to effect a timely response, and please charge any deficiency in fees or credit any overpayments to Deposit Account No. 05-1323 (Docket #010331.49927US).

Respectfully submitted,

January 24, 2005

Kening Li, Ph.D.
Registration No. 44,872
J. D. Evans
Registration No. 26,269

CROWELL & MORING LLP
Intellectual Property Group
P.O. Box 14300
Washington, DC 20044-4300
Telephone No.: (202) 624-2500
Facsimile No.: (202) 628-8844
345383v1

1: Arch Biochem Biophys. 1999 Sep 1;369(1):24-9. Related Articles, Links

How similar are P450s and what can their differences teach us?

Graham SE, Peterson JA.

Department of Biochemistry, University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Boulevard, Dallas, Texas, 75235-9038, USA.

Cytochromes P450 form a very large superfamily of proteins which metabolize substrates from steroids to fatty acids to drugs and are found in organisms from protists to mammals. P450s all appear to take on a similar structural fold, yet frequently having less than 20% sequence identity and having vastly different substrates. Within the structural fold there appears to be a highly conserved core, as determined from the comparison of the structures of the six crystallized, soluble P450s. There are also variable regions which by and large appear to be associated with substrate recognition, substrate binding, and redox partner binding. Molecular dynamics simulations of motion in P450cam and P450BM-3 indicate that substrate binding and product release require substantial motion around the "substrate access channel." Additionally, at the 11th International Conference on Cytochrome P450 Biochemistry, Biophysics, and Molecular Biology and briefly here, the first structure of a microsomal eukaryotic P450 will be presented and compared to the already determined structures by Drs. Johnson and McRee. Finally, with a better understanding of the structure/function relationship of P450s, one will be better able to modify P450s to metabolize the substrates of choice or produce needed valuable chemicals.

Publication Types:
- Review
- Review, Tutorial

PMID: 10462437 [PubMed - indexed for MEDLINE]

Display Abstract Show: 20 Sort Send to Text

Jan 12 2005 06:52:28

VOLUME 18, NO. 1 2001

DRUG METABOLISM AND DRUG INTERACTIONS

Managing Editor: NAOMI KINGSLEY

Freund Publishing House (U.K.) Ltd.
Suite 500, Chesham House
150 Regent Street
London W1R 5FA
England

ISSN 0334-2190

ROLES OF CYTOCHROME P450 IN DEVELOPMENT

Ivaylo Stoilov[1], Ingela Jansson[2], Mansoor Sarfarazi[1] and John B. Schenkman[2]*

Departments of [2]Pharmacology and [1]Surgery, University of Connecticut Health Center, Farmington, CT 06030, USA

CONTENTS

*Author for correspondence
e-mail: jschenkm@neuron.uchc.edu

SUMMARY

Cytochrome P450 (CYP) forms are ubiquitous in nature, appearing in almost all phyla, with many forms appearing in any organism. About 50 different forms have been identified in man, and some of these are found in the embryo, some showing temporal dependence. Many of the forms of cytochrome P450 present in one species have homologues in other species. For example, CYP1A2 is present in many species, including man, rabbits, rodents, fish and fowl. The amino acid sequence identity of these homologues is often in excess of 70%. CYP26, too, has more than 61% identity in amino acid sequence between fish, fowl and mammals. In view of the high degree of conservation of sequence as well as of enzymatic activities, it is only reasonable to assume that such strong conservation of sequence also reflects a conservation of function. Since the 'xenobiotic metabolizing' enzymes predate the production of the many xenobiotics they are known to metabolize, perhaps it is reasonable to consider endobiotics as natural substrates for their metabolism. Of the identified forms of cytochrome P450 that are present in embryonic tissue, we consider the possibility that they serve the organism in support of morphogenesis of the embryonic tissue. These forms may either function to generate morphogenic molecules or to keep regions free of them, thereby creating temporal and spatial regions of morphogen action and supporting region-specific changes in cells. One known morphogen, retinoic acid, has the enzymes retinal dehydrogenase (RALDH) and CYP26 maintaining its actions, the former responsible for its generation and the latter for its elimination. Another form of cytochrome P450, CYP1B1 appears also to be involved in differentiation of tissue, with its absence resulting in primary congenital glaucoma. However, the nature of the morphogen it may maintain still remains to be elucidated.

KEY WORDS

cytochrome P450, embryogenesis, developmental regulation, morphogen metabolism

1. INTRODUCTION

The recognition of the existence of cytochrome P450 hemoproteins dates back to the late 1950s, when a carbon-monoxide-binding pigment was reported to be present in the endoplasmic reticulum of liver /1,2/, and to the later identification of the pigment as a b-type cytochrome /3-5/. Shortly thereafter, the ability of cytochrome P450 to serve as a terminal oxidase in the metabolism of steroids /6/ and xenobiotics /7/ was demonstrated. These observations were quickly followed by the recognition that multiple forms of cytochrome P450 exist in the fragments of endoplasmic reticulum, the microsomes /8-12/.

Studies demonstrating the importance of the different cytochromes P450 in metabolism of drugs and chemicals, and in the activation of various toxicants, teratogens and carcinogens quickly followed /13-19/. Attention subsequently turned to human polymorphisms in cytochrome P450 forms, and their effects on xenobiotic metabolism /20-28/. The importance of the cytochrome P450 enzymes with respect to development of new therapeutic agents was immediately recognized and considerable resources are currently devoted to the interactions of these agents with the different forms of cytochrome P450. Newly developed chemicals being considered for use as drugs are routinely examined for metabolism by different forms of human cytochrome P450, since these represent the major routes of elimination from the body, and metabolites are routinely screened for pharmacological activities. The focus of studies on drug metabolism and xenobiotic activation has resulted in inertia in inquiries as to whether endogenous substrates of cytochrome P450 exist and how such substrates or their metabolites might influence physiological functions. Our objective in the present paper is the review of the literature with respect to our hypothesis that specific members of the cytochrome P450 superfamily may exist which have a role in normal development. By "development" we mean the basic biological phenomena occurring during the generation of a multicellular organism from a single fertilized egg: cell division, pattern formation, morphogenesis, cell differentiation and growth. Such cytochrome P450s might also be capable of *in vitro* metabolism of xenobiotics, but their appearance in the developing embryo at a specific stage of development would suggest a specific role in the development of the organism or of a specific tissue.

Our argument is based on three main elements:

1. Genetic studies have established linkage between P450 mutations and developmental defects.
2. In the developing embryo, a number of cytochrome P450 forms are expressed in extrahepatic tissues undergoing morphogenic transformations.
3. Some cytochrome P450 forms have been shown to be involved in the metabolism of signaling molecules essential for normal development.

2. CURRENT STATUS AND NOMENCLATURE

At present the superfamily of cytochrome P450 consists of about 1200 individual genes, including some 310 mammalian forms, according to the Russian cytochrome P450 database (http://cpd.ibmh.msk.su/online/main/htm). This includes forms in species of all phyla examined, from bacteria to yeast and other primitive eukaryotes to simple plants and trees. There are 17 mammalian families of cytochrome P450. The greatest variability in number of members of the subfamilies lies in families 2, 3 and 4, which contain the endoplasmic reticulum enzymes of xenobiotic metabolism. For example, family 2 has 10 subfamilies (14 if non-mammalian species are included). At present 52 different forms of cytochrome P450 have been identified for the human genome (Fig. 1). Fifteen of these forms are in family 2, four are in family 3 and 11 are in family 4. Thus, more than half of cytochrome P450 families contain single members, each presumably effecting a specific task related to homeostasis in the organism. A number of the forms of cytochrome P450 are present in many species as orthologous proteins. That is, they have at least 45% identity of amino acids in alignments between species (and many have greater than 90% identity) and catalyze the same reaction *in vivo*. These forms are given the same designation, e.g. CYP1B1, or CYP51, the latter an enzyme used in synthesis of cholesterol (or ergosterol in fungi), in the different species. In view of the very large number of forms of cytochrome P450 that exist and the high degree of sequence identity between orthologous forms in different species, it is difficult to consider that these enzymes have developed just to oxidize the drugs and chemicals developed by man in the last couple of centuries. It is possible that very similar orthologous cytochrome P450 enzymes have

retained their functions during evolution over the eons in maintenance of homeostasis and in aid of development of the organism. Below we discuss the functions of cytochrome P450 and its putative roles in the biology of development.

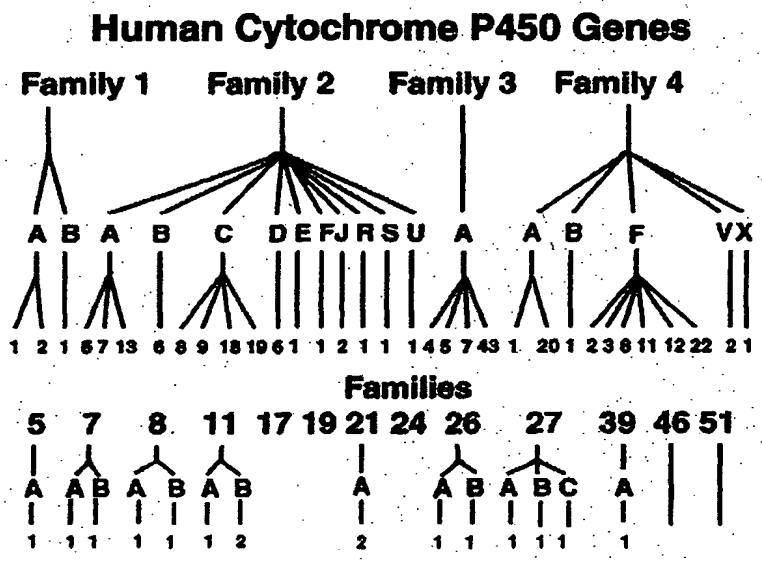

Fig. 1: Human cytochrome P450 genes. The families of cytochrome P450 (CYP) are designated by an Arabic numeral, e.g. CYP1. At present about 157 families are known. Subfamilies are designated by a letter, e.g. CYP1A, and members in the subfamilies by an Arabic numeral, e.g. CYP1A2. Thus, family 1 consists of two subfamilies, CYP1A and CYP1B. CYP1A contains two members, CYP1A1 and CYP1A2, while subfamily 1B only contains one member, CYP1B1. Most of the families of cytochrome P450 contain one or two members, and these often have orthologous forms in other species, e.g. CYP51 is present in fungi, mammals, plants, etc., indicating a high degree of specificity in its function in biological processes.

3. CYTOCHROME P450-PRODUCTION OF CHEMICAL MEDIATORS

3.1 Molecular structure

Cytochrome P450 proteins have an average mass of approximately 50 kDa, and all have an iron protoporphyrin IX (heme) prosthetic group liganded to a cysteine thiolate. All appear to be membrane proteins, with the exception of several bacterial forms. Structurally the proteins are anchored to the endoplasmic reticular membrane or the inner mitochondrial membrane by a transmembrane amino terminus. The very hydrophobic amino-terminal region of the protein contains a membrane insertion sequence as well as a stop-transfer sequence that functions as an anchor in the membrane determining the topological orientation of the cytochrome P450 /29-31/. This hydrophobic region is followed by a proline-rich 'hinge' region, which imparts flexibility between the transmembrane region and the globular catalytic part of the protein that resides in the cytosolic region of the cell or oriented toward the mitochondrial matrix. This flexibility may be necessary to orient the cytosolic portion of the molecule with respect to the membrane for substrate access and for interaction with the appropriate electron transfer partner. At least one of the bacterial forms (CYP102) and one of the mammalian forms of cytochrome P450 (NOS-I) exist as a fusion protein with an electron transfer partner, NADPH-cytochrome P450 reductase /32-36/. The carboxyl-terminal portion of the cytochrome P450 consists of a conserved core structure shared by all members of the cytochrome P450 superfamily /37/. These structures include a number of α-helices and β-sheets and a 'meander' region, all necessary for the proper structural orientation of the heme prosthetic group /37-39/ that makes this family of enzymes mono-oxygenases. Interestingly, as noted below, mutations affecting these structures in CYP1B1 result in abnormal eye development.

3.2 Biochemistry of cytochrome P450

The cytochromes P450 are monooxygenases. They accept two reducing equivalents sequentially and use these to reduce molecular oxygen to an oxidizing species that forms one molecule of water and one oxidized substrate molecule. The monooxygenase reaction can be described by the equation:

$$O_2 + 2e^- + SH + 2H^+ \longrightarrow H_2O + SOH$$

where SH is the substrate to be oxidized and SOH is the oxidized substrate. The types of reactions catalyzed by the different forms of cytochrome P450 are, perhaps, more varied than any other enzyme /40/. Different cytochromes P450 can hydroxylate aliphatic and aromatic carbons and form epoxides across double bonds. They can also remove alkyl groups from nitrogen, oxygen or sulfur atoms by inserting oxygen onto the alkyl moiety. Some are also capable of removing and replacing nitrogen and sulfur in molecules with oxygen, and of the formation of double bonds (dual hydrogen atom abstraction).

3.3. Substrates of cytochrome P450

In considering roles for cytochrome P450 forms in development and in maintenance of homeostasis in organisms it is helpful to recognize that while they may metabolize a wide variety of compounds foreign to the body (xenobiotics), *in vivo* these enzymes may utilize a specific endogenous substrate. Perhaps they generate a stereospecific metabolite targeting a specific receptor. However, members of cytochrome P450 families 1, 2, 3 and 4 are called xenobiotic metabolizing enzymes, and oxidize a large number of lipophilic drugs and chemicals of varying shapes and sizes (Fig. 2) as well as a number

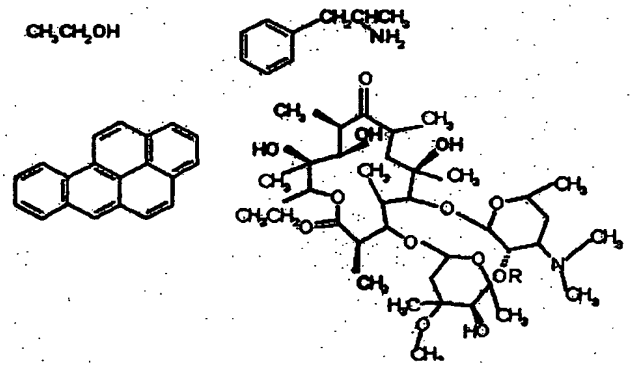

Fig. 2: Some xenobiotic substrates of cytochrome P450. Structures of ethanol, amphetamine, benzo[*a*]pyrene and erythromycin are shown. These are oxidized to the aldehyde, deaminated, oxidized to hydroxy or epoxide metabolite, and *N*-dealkylated, respectively.

of compounds of intermediary metabolism (endobiotics). It was suggested that these enzymes function to decrease the lipid/water partition coefficient of the xenobiotics and endogenous compounds and thereby make them more readily excreted by the kidneys /41/. Substrates range in size from a mass of 36 Da (ethanol), to planar, aromatic polycyclic hydrocarbons such as benzo[*a*]pyrene (252 Da), to large macrolide compounds such as erythromycin (734 Da). The substrate specificities of the different forms of cytochrome P450 are broad and overlapping. However, most will metabolize a number of physiologically relevant compounds of intermediary metabolism, producing different products. For example, the duration of action of a series of barbiturates was shown to be inversely related to their partition coefficients and to their rates of metabolism /40/. As with xenobiotics, elimination of lipophilic waste compounds of endogenous metabolism is also enhanced through oxidation by cytochrome P450 forms. For example, steroids are eliminated as multiple hydroxylated metabolites and their conjugates /42/.

In contrast to the many forms of cytochrome P450 with broad overlapping substrate specificities, many cytochrome P450 forms in other families participate in fairly specific biosynthetic reactions, generating products involved in the homeostasis of the organism. Such forms generally exist in families with only one or two members. Examples include CYP51, involved in formation of cholesterol, phytosterol or ergosterol, CYP27A, which produces bile acids, CYP11A1, which forms progestanes, CYP11B and CYP21, which generate corticosteroids, CYP17, for production of androgens, CYP19 for production of estrogens, CYP2D25 for 25-hydroxyvitamin D_3 activation, and CYP26 which catalyzes catabolism of all-*trans* retinoic acid.

4. STUDIES IMPLICATING CYTOCHROME P450 IN TISSUE DEVELOPMENT

As indicated earlier, evidence has begun to appear that provides an indication that a number of different forms of cytochrome P450 may be involved in development of the organism. Such evidence includes genetic linkage between cytochrome P450 mutations that result in developmental defects, the discrete temporal and spatial localization of different forms of cytochrome P450 in extrahepatic embryonic

tissues, and the identification of the involvement of cytochrome P450 in the metabolism of signaling molecules essential for normal embryonic development.

4.1 Genetic studies link CYP1B1 to developmental eye disorder - primary congenital glaucoma

Genetic linkage analysis of families with primary congenital glaucoma (PCG) identified two chromosomal loci linked to the disease phenotyope - GLC3A on chromosome 2p21 and GLC3B on chromosome 1p36 /43,44/. Efforts to clone the PCG gene residing in locus GLC3A indicated that the gene mutated in individuals with PCG was a cytochrome P450, CYP1B1 /45/, and was demonstrated by quantitative PCR and Northern blot analysis. The genetic linkage of CYP1B1 defects and PCG has been confirmed by similar findings in other laboratories /46,47/. A total of 23 different mutations were shown to segregate with the PCG disease phenotype in affected families and not to be polymorphisms found in the general population /48/. A number of these mutations reported in patients with PCG were mapped against a 3D model of the CYP1B1 molecule constructed by homology modeling /49/. The missense mutations were found to affect highly conserved amino acid residues located predominantly either in the hinge region or the Conserved Core Structures (CCS) /50/ of the CYP1B1 molecule. These mutations therefore are expected to interfere with fundamental properties of the cytochrome P450 molecule, such as proper folding, heme binding, substrate accommodation and interaction with the redox partner. Another group of mutations was predicted to introduce premature stop codons by frameshifts in the CYP1B1 open reading frame. These mutations would eliminate at least the heme-binding region of CYP1B1, which is essential for the normal function of every P450 molecule. Therefore, it is expected that these mutations would result in functional null alleles.

How might these defects translate to PCG? Cytochrome P450 has been shown to be present in bovine eye /51/, and cytochrome P450 metabolic activities were shown to differ in different regions of the eye /52/. As shown in Figure 3, during normal eye development the trabecular meshwork cells are shifted from below the iris junction to a position above that junction to a point where it connects to the anterior chamber of the eye. From that position it can serve the function of filtering the anterior chamber fluid for drainage of that chamber. In

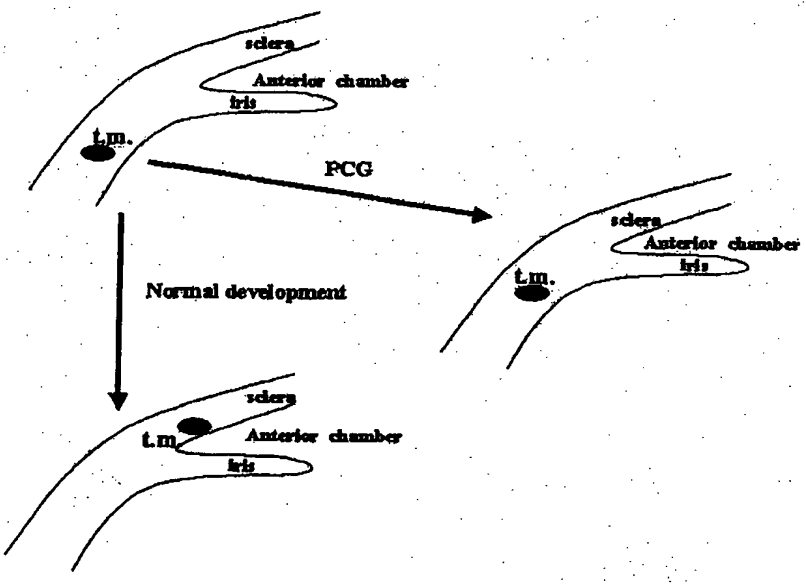

Fig. 3: Scheme of the movement of the trabecular meshwork (t.m.) in the formation of the eye during embryonic development.

PCG the development of that region of the eye is arrested at about that of the 7 month fetus at the time of birth, and pressure in the anterior chamber begins to rise even prior to birth /53/. Two possible scenarios on how CYP1B1 mutations may trigger pathogenic responses resulting in abnormal eye development are: 1) The spatial and temporal expression of genes controlling the anterior chamber angle development may be altered by the absence of a regulatory molecule (such as steroid or lipid metabolite) produced by CYP1B1. 2) Alternatively, the signs of developmental arrest may reflect the toxic effect of a metabolite that is normally eliminated by CYP1B1.

A CYP1B1-null mouse strain has been constructed in which the homozygous animals were reported not to show any evidence of glaucoma /54/. Unfortunately, the methods used to evaluate the mouse (gross examination and standard behavioral comparisons) may not be sensitive enough to detect glaucomatous changes in the mouse eye /55/. In addition, the mouse phenotype may differ from the human, since the anterior chamber angle has undergone some very recent evolutionary changes. For example, only humans and higher apes have

the typical trabecular-type meshwork, while reticular-type meshwork is present in lower organisms.

4.2. Expression of cytochrome P450s in embryonic tissues undergoing morphogenic transformation

Genetic studies mentioned implicate cytochrome P450 in control of normal development. The next step in identifying its role in morphogenic conversion would be to demonstrate that cytochrome P450 is present in embryonic tissue undergoing morphogenic transformation. Numerous studies have reported the presence of individual forms of cytochrome P450 in the developing embryo. Early studies on the involvement of cytochrome P450 in embryogenesis and tissue development demonstrated the presence of mRNA of NADPH-cytochrome P450 reductase and CYP51 in the 4-day (preimplantation) mouse blastocyst. Specific tests for other forms of cytochrome P450 involved in steroid metabolism, CYP17, CYP11A1, CYP19 and CYP27, were negative /56/. In another study, CYP1A1 and CYP1A2 were not found in the rabbit fetus, while CYP3A6 appeared on day 30, the last day prior to birth /57/. In mouse fetus, mRNA encoding CYP2B19 appears in developing keratinocytes in the upper skin layer on day 15 /58/. The distribution of this mRNA was specific to the fetal mouse epidermis. The temporal appearance of this enzyme during initiation of the epidermal stratification suggests a possible involvement in this function. The recombinant protein expressed in *Escherichia coli* was capable of arachidonate metabolism, forming two metabolites, 11,12-epoxyeicosatrienoic acid and 14,15-epoxyeicosatrienoic acid, also found in murine skin. Other metabolites were also found in the *in vitro* assays. An orthologous form of cytochrome P450, CYP2B15, found in rat, has 86% sequence identity to CYP2B19, and like it is specific to keratinocytes /58/.

While these studies have established that cytochrome P450 forms are present in the developing embryo, studies on CYP26 have provided a model for investigating the relevance of cytochrome P450 expression during development. *In situ* hybridization analysis of the spatio-temporal pattern of expression of this orthologous form of cytochrome P450 in the various species demonstrates a relevance of its expression to the pattern of development (see below). The high degree of sequence identity of the CYP26 protein between orthologous forms in different species suggests that the protein is carrying out a

function important for the development of the embryo. For example, the degree of sequence identity between the human and mouse forms of this enzyme is 93%, and the sequence identity between human and zebrafish is 68%. *Xenopus laevis* CYP26 was reported to have 68% amino acid identity to the mouse enzyme /59/. CYP1B1, as indicated above, has similarly been shown to be expressed in the developing eye (mouse) in a pattern consistent with its proposed function as regulator of the anterior chamber angle development. In the case of CYP1B1, the sequence identity between mouse and rat is 93%, and between rat and human 80%. In agreement with a role for the cytochrome P450 monooxygenases in embryogenesis and development is the observation that the electron transfer protein, NADPH-cytochrome P450 reductase, necessary for the proper function of endoplasmic reticular forms of cytochrome P450, also shows differential tissue distribution in developing embryos /60/.

4.3. Cytochrome P450 functions in morphogen metabolism

In the present review we consider the role of cytochrome P450 forms in the development of the organism. Our goal is to provide information supporting a role for this family of enzymes, suggesting forms functioning in morphogenesis and development make use of small molecules (morphogens), which they synthesize or destroy, in support of the gradient concept, as discussed by Crick /61/:

"...One postulates a source - a cell which produces the chemical (which I shall call a morphogen) and maintains it at a constant level. At the other end the extreme cell acts as a sink: that is, it destroys the molecule..." "I doubt if morphogens will turn out to be large proteins or common ions like K^+ or Na^+. The obvious choice would be an organic molecule of the size of, say, cyclic AMP or a steroid. That is, with the molecular weight in the range of 300 to 500."

That vitamin A (retinol) is a requirement for normal embryonic development has been known for almost 75 years. It is a small molecule (Fig. 4) of mass 294 and consists of a benzene ring coupled to a linear 9 carbon polyene sidechain. Its absence results in structural defects, tissue morbidities such as defective heart, nervous system, urogenital system, and eye, in the embryo and newborn animal, and embryonic lethality if the deficiency is severe enough. Similar effects are seen in retinoid receptor-null mutants /62/. Retinol is obtained in

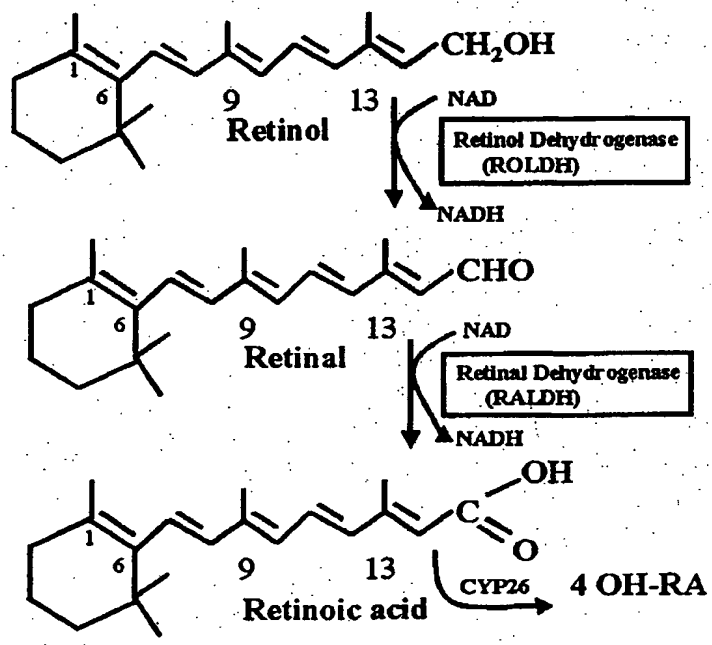

Fig. 4: Steps in the metabolism of vitamin A (retinol) to retinoic acid *in vivo* and the enzymes involved.

the diet from animal sources and β-carotene, a bis-retinal compound that yields retinal on oxidation *in vivo*, is obtained from plant sources. Retinol dehydrogenase, an enzyme distinct from alcohol dehydrogenase, converts the retinol to retinal whereupon another distinct enzyme, retinal dehydrogenase-2 (RALDH-2), converts it to retinoic acid, the active compound /63/. In studies with RALDH-2-null mice it could be shown that embryos negative for RALDH-2 generally die at mid-gestation /63/. At least two retinoid receptor families exist in the nucleus and serve as ligand-activated transcriptional regulators. These include the all-*trans* and 9-*cis* retinoic acid activated receptors (RAR) and the retinoid X-receptor (RXR), which is only activated by 9-*cis* retinoic acid, which bind to a retinoic acid response element in the promoter region of target genes /64/. Each of the two receptor family types consists of three isotypes, α, β, and γ /65/. Two cytoplasmic binding proteins (CRABP I and CRABP II) exist which have been

suggested to have a modulating effect on retinoic acid levels reaching the nucleus /64/. While retinoic acid has been unequivocally demonstrated to have positive influences on the development of specific regions of the embryo, based upon teratogenic effects of pharmacological levels applied, abnormalities resulting from deficiency, and on RAR^- and RXR^- (null) constructs, in order to be considered a morphogen another criterion must be met, i.e. the existence of a sink. Such a sink was discovered in the form of CYP26 /66/, a form of cytochrome P450 that specifically catabolizes retinoic acid to the less effective 4-hydroxyretinoic acid metabolite (Fig. 4). CYP26 and RALDH-2 provide the necessary functions that make retinoic acid a morphogen. They are differentially and exclusively distributed in the embryo, with levels both temporally and spatially distinct (Fig. 5). Their distinctly regionalized and nonoverlapping boundaries of distribution create a morphogenic gradient of retinoic acid that explains the head-to-tail axis formation /67/. As shown in Figure 5, retinoic acid is generated by RALDH in the posterior region of the embryo and its diffusion is curtailed by CYP26 in the anterior portion of the embryo, resulting in a sharp boundary between areas impacted by retinoic acid and areas not impacted by the agent.

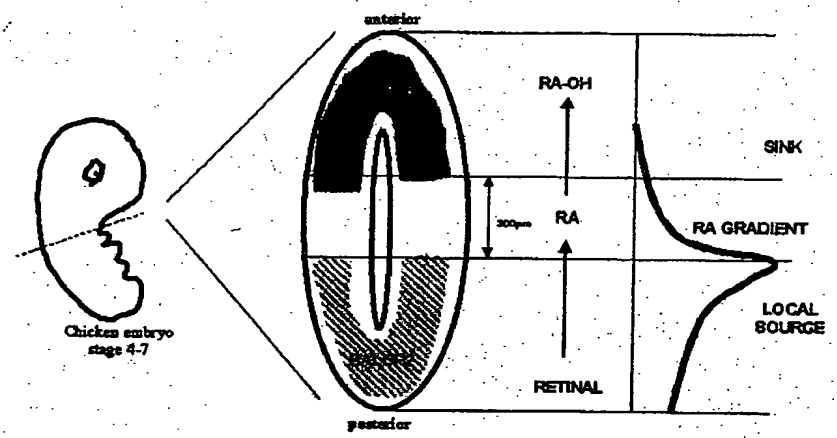

Fig. 5: Scheme depicting the retinoic acid gradient serving as a morphogen in the developing embryo.

Interestingly, a number of other forms of human cytochrome P450 have been identified that can also 4-hydroxylate retinoic acid /68/.

These include CYP2C8, CYP3A4, and CYP2C9. This is not unexpected, in view of the broad, overlapping spectrum of substrates of cytochrome P450 forms. However, these enzymes do not appear to be expressed in the developing embryo and thus do not appear to be involved in morphogenesis. Based upon inhibition of *trans* retinoic acid 4-hydroxylation in human fetal liver by the CYP3 inhibitor, troleandomycin, it was suggested that CYP3A7 plays a role in detoxifying this compound /69/. In contrast, based upon lack of effect of specific inhibitors, it was concluded /69/ that hepatic CYP1A1, CYP1A2, CYP1B1, CYP2C8 and CYP2E1 did not metabolize retinoic acids. At present, only CYP26, like CYP1B1, has been shown clearly to have a role in morphogenesis. However, discovery of CYP26 followed establishment of retinoic acid as a morphogen. In the case of CYP1B1, the opposite is true. The involvement of CYP1B1 in normal eye development was discovered by genetic linkage. The more difficult task that remains is to determine the nature of the morphogen involved in normal eye development and influenced by CYP1B1.

5. EXAMINATION OF PUTATIVE MORPHOGENIC AND PROMORPHOGENIC SUBSTRATES OF CYP1B1

If cytochrome P450 forms are responsible for morphogenic activity, we would expect the promorphogen or morphogen to be a small molecule of lipophilic nature, i.e. a molecule with characteristics similar to that of other known cytochrome P450 substrates. Examples of some known cytochrome P450 substrates and metabolites are shown in Figure 6. With the exception of nitric oxide synthetase (e.g. NOS-1), all of the forms of cytochrome P450 metabolize lipophilic compounds. Substrates include fatty acids, producing ω- and (ω-1)-hydroxylation products /58,70-73/, prostaglandins, yielding ω- to (ω-2)-hydroxylation metabolites /74-76/, and leukotriene ω- and (ω-1)-hydroxylation products /77/. Other metabolites of endogenous substrates include isomeric and epimeric-specific androgen, estrogen and progestane hydroxylation products /78-85/. The many potential endogenous substrates of the different cytochrome P450 forms and their potential metabolites, that may be involved in morphogen formation, make it difficult to predict what may or may not be a morphogen, as in the case of the CYP1B1-deficiency PCG phenotype. However, continued studies on the substrate specificity and metabolite profile of

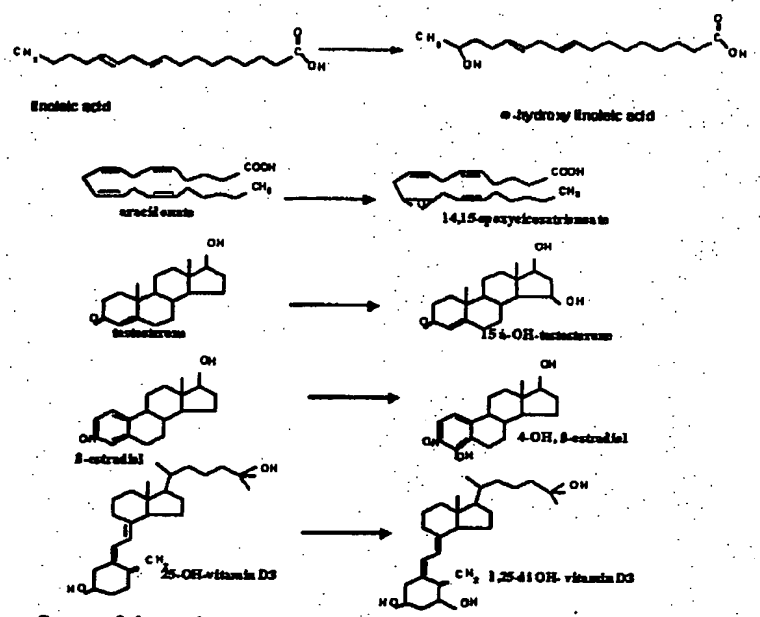

Fig. 6: Some of the endogenous (endobiotic) substrates of cytochrome P450. All of the substrates are converted to numerous metabolites by the different forms of cytochrome P450, but only a single metabolite of each is shown.

this enzyme may yield information, perhaps identifying the specific agent serving as morphogen.

From the data reviewed, it appears that different forms of cytochrome P450 are present in different regions of the adult eye, and in the developing eye tissue, based upon differences in location of xenobiotic metabolizing activities and NADPH-cytochrome P450 reductase. It was hypothesized that metabolites of cytochrome P450 forms might have physiological functions. Indeed, the ciliary body of the eye was seen to have the greatest xenobiotic metabolizing activity, followed by the retinal pigment epithelium /52/. An extension of this hypothesis was the proposal that the development of the different "drug metabolizing (P450) enzymes" from early evolutionary forms might relate to their ability to utilize endogenous substrates necessary for the regulation of processes of growth and differentiation /86/.

6. CONCLUSIONS

Cytochrome P450 forms are very strong suspects as potential players in the development of the organism. Because of their great diversity in both substrate recognition and stereochemical diversity in product production, they are strong candidates for morphogen production. Which form of cytochrome P450 appears in a tissue at a particular time will determine what will be produced from a particular endogenous substrate that presents itself at that time. Depending upon the form of cytochrome P450 that appears, the endogenous substrate may be converted to a metabolite for elimination or to a morphogen, or activated to a teratogen or carcinogen. At least one instance, the conversion of the metabolite retinoic acid to a less active 4-hydroxy-retinoic acid, enables developmental changes during embryonic development. In another instance, that of eye development, absence of CYP1B1 causes cessation of the trabecular meshwork development in the eye and results in PCG. Studies are currently underway to determine the nature of the morphogen in normal eye development.

ACKNOWLEDGEMENTS

This report was supported in part by grants from The Glaucoma Foundation and NIH grants 1R01 EY 11095 and 2 R01 ES03154.

REFERENCES

1. Garfinkel D. Studies on pig liver microsomes. I. Enzymic and pigment composition of different microsomal fractions. Arch Biochem Biophys 1958; 77: 493-509.
2. Klingenberg M. Pigments of rat liver microsomes. Arch Biochem Biophys 1958; 75: 376-386.
3. Omura T, Sato R. A new cytochrome in liver microsomes. J Biol Chem 1962; 237: 1375-1376.
4. Omura T, Sato R. The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purification, and properties. J Biol Chem 1964; 239: 2379-2385.
5. Omura T, Sato R. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J Biol Chem 1964; 239: 2370-2378.
6. Estabrook RW, Cooper DY, Rosenthal O. The light reversible carbon monoxide inhibition of the steroid C21-hydroxylase system of the adrenal cortex. Biochem Z 1963; 338: 741-755.

7. Cooper DY, Levin S, Narasimhulu S, Rosenthal O, Estabrook RW. Photochemical action spectrum of the terminal oxidase of mixed function oxidase systems. Science 1965; 147: 400-402.
8. Sladek NE, Mannering GJ. Evidence for a new P-450 hemoprotein in hepatic microsomes from methylcholanthrene treated rats. Biochem Biophys Res Commun 1966; 24: 668-674.
9. Alvares AP, Schilling G, Levin W, Kuntzman R. Studies on the induction of CO-binding pigments in liver microsomes by phenobarbital and 3-methylcholanthrene. Biochem Biophys Res Commun 1967; 29: 521-526.
10. Thomas PE, Lu AYH, Ryan D, West SB, Kawalek J. Multiple forms of rat liver cytochrome P-450. Immunochemical evidence with antibody against cytochrome P-448. J Biol Chem 1976; 251: 1385-1391.
11. Lu AYH, Kuntzman R, West S, Conney AH. Reconstituted liver microsomal enzyme system that hydroxylates drugs, other foreign compounds and endogenous substrates. I. Determination of substrate specificity by the cytochrome P-450 and P-448 fractions. Biochem Biophys Res Commun 1971; 42: 1200-1206.
12. Guengerich FP. Isolation and purification of cytochrome P-450, and the existence of multiple forms. In: Schenkman JB, Kupfer D, eds. Hepatic Cytochrome P-450 Monooxygenase System. New York: Pergamon Press, International Encyclopedia of Pharmacology and Therapeutics 1982; 108: 497-521.
13. Niranjan BG, Avadhani NG. Activation of aflatoxin B_1 by a mono-oxygenase system localized in rat liver mitochondria. J Biol Chem 1980; 255: 6575-6578.
14. Peng R, Tennant P, Lorr NA, Yang CS. Alterations of microsomal monooxygenase system and carcinogen metabolism by streptozotocin-induced diabetes in rats. Carcinogen 1983; 4: 703-708.
15. Potter DW, Levin W, Ryan DE, Thomas PE, Reed DJ. Stereoselective monooxygenation of carcinostatic 1-(2-chloroethyl)-3-(cyclohexyl)-1-nitrosourea and 1-(2-chloroethyl)-3-(trans-4-methylcyclohexyl)-1-nitrosourea by purified cytochrome P-450 isozymes. Biochem Pharmacol 1984; 33: 609-613.
16. Guengerich FP, Shimada T, Distlerath LM, Reilly PEB, Umbenhauer DR, Martin MV. Human cytochrome P-450 isozymes: polymorphism and potential relevance in chemical carcinogenesis. In: Harris C, ed. Biochemical and Molecular Epidemiology of Cancer. New York: Alan R. Liss, Inc., 1986; 205-211.
17. Guengerich FP. Catalytic selectivity of human cytochrome P450 enzymes: relevance to drug metabolism and toxicity. Toxicol Lett 1994; 70: 133-138.
18. Gonzalez FJ, Gelboin HV. Role of human cytochromes P450 in the metabolic activation of chemical carcinogens and toxins. Drug Metab Rev 1994; 26: 165-183.
19. Nebert DW. Role of genetics and drug metabolism in human cancer risk. Mutation Res 1991; 247: 267-281.
20. Idle JR, Smity RL. Polymorphisms of oxidation at carbon centers of drugs and their clinical significance. Drug Metab Rev 1979; 9: 301-317.

21. Johnson EF, Schwab GE. Constitutive forms of rabbit-liver microsomal cytochrome P-450: enzymatic diversity, polymorphism and allosteric regulation. Xenobiotica 1984; 14: 3-18.
22. Guengerich FP, Umbenhauer DR, Churchill PF, Beaune PH, Bocker R, Knodell RG, Martin MV, Lloyd RS. Polymorphism of human cytochrome P-450. Xenobiotica 1987; 17: 311-316.
23. Wrighton S, Ring B, Watkins P, Vandenbranden M. Identification of a polymorphically expressed member of the human cytochrome P-450III family. Mol Pharmacol 1989; 36: 97-105.
24. Meyer UA. Genetic polymorphisms of drug metabolism. Fund Clin Pharmacol 1990; 4: 595-615.
25. Yasumori T, Yamazoe Y, Kato R. Cytochrome-P-450 human-2 (P-450IIC9) in mephenytoin hydroxylation polymorphism in human livers. Differences in substrate and stereoselectivities among microheterogeneous P-450IIC species expressed in yeasts. J Biochem 1991; 109: 711-717.
26. Holsztynska EJ, Weber WW, Domino EF. Genetic polymorphism of cytochrome P-450-dependent phencyclidine hydroxylation in mice. Comparison of phencyclidine hydroxylation in humans. Drug Metab Dispos 1991; 19: 48-53.
27. Kato S, Shields PG, Caporaso NE, Hoover RN, Trump BF, Sugimura H, Weston A, Harris CC. Cytochrome-P450IIE1 genetic polymorphisms, racial variation, and lung cancer risk. Cancer Res 1992; 52: 6712-6715.
28. Persson I, Johansson I, Bergling H, Dahl ML, Seidegard J, Rylander R, Rannug A, Hogberg J, Sundberg MI. Genetic polymorphism of cytochrome-P4502E1 in a Swedish population. Relationship to incidence of lung cancer. FEBS Lett 1993; 319: 207-211.
29. Sakaguchi M, Mihara K, Sato R. Signal recognition particle is required for cotranslational insertion of cytochrome P-450 into microsomal membranes. Proc Natl Acad Sci USA 1984; 81: 3361-3364.
30. Sakaguchi M, Mihara K, Sato R. A short amino-terminal segment of microsomal cytochrome P-450 functions both as an insertion signal and as a stop-transfer sequence. EMBO J 1987; 6: 2425-2431.
31. Sakaguchi M, Tomiyoshi R, Kuroiwa T, Mihara K, Omura T. Functions of signal and signal-anchor sequences are determined by the balance between the hydrophobic segment and the N-terminal charge. Proc Natl Acad Sci USA 1992; 89: 16-19.
32. Stuehr DJ, Ikedasaito M. Spectral characterization of brain and macrophage nitric oxide synthases. Cytochrome-P-450-like hemeproteins that contain a flavin semiquinone radical. J Biol Chem 1992; 267: 20547-20550.
33. Bredt DS, Hwang PM, Glatt CE, Lowenstein C, Reed RR, Snyder SH. Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. Nature 1991; 351: 714-718.
34. White K, Marletta M. Nitric oxide synthase is a cytochrome-P-450 type hemoprotein. Biochemistry 1992; 31: 6627-6631.
35. Black SD. On the domain structure of cytochrome P450 102 (BM-3): isolation and properties of a 45-kDa FAD/NADP domain. Biochem Biophys Res Commun 1994; 203: 162-168.

36. Klein ML, Fulco AJ. Critical residues involved in FMN binding and catalytic activity in cytochrome-P450(BM-3). J Biol Chem 1993; 268: 7553-7561.
37. Graham-Lorence SE, Peterson JA. Structural alignments of P450s and extrapolations to the unknown. Meth Enzymol 1996; 272: 315-326.
38. Poulos TL. Modeling of mammalian P450s on basis of P450cam Xray structure. Meth Enzymol 1991; 206: 11-30.
39. Poulos TL, Finzel BC; Howard AJ. High-resolution crystal structure of cytochrome P450cam. J Mol Biol 1987; 195: 687-700.
40. Schenkman JB. The fate of xenobiotics in the body: enzymes of metabolism. In: Arinc E, Schenkman JB, Hodgson E, eds. Molecular and Appplied Aspects of Oxidative Drug Metabolizing Enzymes. New York: Plenum Publishers, Nato ASI Series A: Life Sci 1999; 303: 1-20.
41. Brodie BB, Gillette JR, La Du BN. Enzymatic metabolism of drugs and other foreign compounds. Ann Rev Biochem 1958; 27: 427-454.
42. Gustafsson BE, Gustafsson J.-A., Sjovall J. Steroids in germfree and conventional rats. 3. Solvolyzable sterol conjugates in germfree and conventional rat faeces. Eur J Biochem 1968; 4: 574-577.
43. Sarfarazi M, Akarsu AN, Hossain A, Turacli ME, Aktan SG, Barsoumn-Homsy M, Sayli S. Assignment of a locus (GLC3A) for primary congenital glaucoma (buphthalmos) to 2p21 and evidence for genetic heterogeneity. Genomics 1995; 30: 171-177.
44. Akarsu AN, Turacli ME, Aktan SG, Barsoum-Homsy M, Chevrette L, Sayli BS, Sarfarazi M. A second locus (GLC3B) for primary congenital glaucoma (buphthalmos) maps to the 1p36 region. Hum Mol Genet 1996; 5: 1199-1203.
45. Stoilov I, Akarsu AN, Sarfarazi M. Identification of three different truncating mutations in cytochrome P4501B1 (CYP1B1) as the principal cause of primary congenital glaucoma (buphthalmos) in families linked to the GLC3A locus on chromosome 2p21. Hum Mol Genet 1997; 6: 641-647.
46. Bejjani BA, Lewis RA, Tomey KF, Anderson KL, Dueker DK, Jabak M, Astle WF, Otterud B, Leppert M, Lupski JR. Mutations in CYP1B1, the gene for cytochrome P450 1B1, are the predominant cause of primary congenital glaucoma in Saudi Arabia. Am J Hum Genet 1998; 62: 325-333.
47. Plasilova M, Stoilov I, Sarfarazi M, Kadasi L, Ferakova E, Ferak V. Identification of a single ancestral CP1B1 mutation in Slovak Gypsies (Roms) affected with primary congenital glaucoma. J Med Genet 1999; 36: 290-294.
48. Sarfarazi M, Stoilov I. Molecular genetics of primary congenital glaucoma. Eye 2000; 14: 422-428.
49. Stoilov I, Akarsu AN, Alozie I, Child A, Barsoum-Homsy M, Turacli ME, Or M, Lewis RA, Ozdemir N, Brice G, Aktan SG, Chevrette L, Coca-Prados M, Sarfarazi M. Sequence analysis and homology modeling suggests that primary congenital glaucoma on 2p21 results from mutations disrupting either the hinge region or the conserved core structures of cytochrome P4501B1. Am J Hum Genet 1998; 62: 573-584.
50. Graham-Lorence S, Peterson JA. P450s: structural similarities and functional differences. FASEB J 1996; 10: 206-214.

51. Schwartzman ML, Abraham NG, Masferrer J, Dunn MW, McGiff JC. Cytochrome P450 dependent metabolism of arachidonic acid in bovine corneal epithelium. Biochem Biophys Res Commun 1985; 132: 343-351.
52. Schwartzman ML, Masferrer J, Dunn MW, McGiff JC, Abraham NG. Cytochrome P450, drug metabolizing enzymes and arachidonic acid metabolism in bovine ocular tissues. Curr Eye Res 1987; 6: 623-630.
53. Anderson DR. The development of the trabecular meshwork and its abnormality in primary infantile glaucoma. Trans Am Ophthalmol Soc 1981; 79: 458-485.
54. Buters JTM, Doehmer J, Gonzalez FJ. Cytochrome P450-null mice. Drug Metab Rev 1999; 31: 437-447.
55. John SWM, Hagaman JR, MacTaggart TE, Peng L, Smithes O. Intraocular pressure in inbred mouse strains. Invest Ophthalmol Vis Sci 1997; 38: 249-253.
56. Stromstedt M, Keeney DS, Waterman MR, Paria BC, Conley AJ, Dey SK. Preimplantation mouse blastocysts fail to express CYP genes required for estrogen biosythesis. Mol Reprod Dev 1996; 43: 428-436.
57. Pineau T, Daujat M, Pichard L, Girard F, Angevain J, Bonfils C, Maurel P. Developmental expression of rabbit cytochrome-P450 CYP1A1, CYP1A2 and CYP3A6 genes. Effect of weaning and rifampicin. Eur J Biochem 1991; 197: 145-153.
58. Keeney DS, Skinner C, Traverse JB, Capdevila JH, Nanney LB, King LE Jr, Waterman MR. Differentiating keratinocytes express a novel cytochrome P450 enzyme, CYP2B19, having arachidonate monooxygenase activity. J Biol Chem 1998; 273: 32071-32079.
59. de Roos K, Sonneveld E, Compaan B, ten Berge D, Durston AJ, van der Saag PT. Expression of retinoic acid 4-hydroxylase (CYP26) during mouse and *Xenopus laevis* embryogenesis. Mech Develop 1999; 882: 205-211.
60. Keeney DS, Waterman MR. Two novel sites of expression of NADPH cytochrome P-450 reductase during murine embryogenesis: limb mesenchyme and developing olfactory neuroepithelia. Develop Dynam 1999; 216: 511-517.
61. Crick F. Diffusion in embryogenesis. Nature 1970; 225: 420-422.
62. Dickman ED, Thaller C, Smith SM. Temporally-regulated retinoic acid depletion produces specific neural crest ocular and nervous system defects. Development 1997; 124: 3111-3121.
63. Niederreither K, Subbarayan V, Dollé P, Chambon P. Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. Nat Genet 1999; 21: 444-448.
64. Morriss-Kay GM, Sokolova N. Embryonic development and pattern formation. FASEB J 1996; 10: 961-968.
65. Mark M, Ghyselinck NB, Wendling O, Dupé V, Mascrez B, Kastner P, Chambon P. A genetic dissection of the retinoid signalling pathway in the mouse. Proc Nutr Soc 1999; 58: 609-613.
66. Fujii H, Sato T, Kaneko S, Gotoh O, Fujii-Kuriyama Y, Osawa K, Kato S, Hamada H. Metabolic inactivation of retinoic acid by a novel P450 differentially expressed in developing mouse embryos. EMBO J 1997; 16: 4163-4173.

67. Maden M. Heads or tails? Retinoic acid will decide. BioEssays 1999; 21: 809-812.
68. McSorley LC, Daly AK. Identification of human cytochrome P450 isoforms that contribute to all-*trans*-retinoic acid 4-hydroxylation. Biochem Pharmacol 2000; 60: 517-526.
69. Chen H, Fantel AG, Juchau MR. Catalysis of the 4-hydroxylation of retinoic acids by CYP3A7 in human fetal hepatic tissues. Drug Metab Dispos 2000; 28: 1051-1057.
70. Bjorkhem I, Hamberg M. On the rate-limiting step in ω-hydroxylation of lauric acid. Biochem Biophys Res Commun 1972; 47: 333-340.
71. Pinot F, Salaun J-P, Bosch H, Lesot A, Mioskowski C, Durst F. ω-Hydroxylation of Z9-octadecenoic, Z9,10-epoxystearic and 9,10-dihydoxystearic acids by microsomal cytochrome P450 systems from *Vicia sativa*. Biochem Biophys Res Commun 1992; 184: 183-193.
72. Lu AYH, Coon MJ. Role of hemoprotein P-450 in fatty acid ω-hydroxylation in a soluble enzyme system from liver microsomes. J Biol Chem 1968; 243: 1331-1332.
73. CaJacob CA, Chan WK, Shephard E, Ortiz de Montellano PR. The catalytic site of rat hepatic lauric acid ω-hydroxylase. Protein versus prosthetic heme alkylation in the ω-hydroxylation of acetylenic fatty acids. J Biol Chem 1988; 263: 18640-18649.
74. Aoyama T, Hardwick JP, Imaoka S, Funae Y, Gelboin HV, Gonzalez FJ. Clofibrate-inducible rat hepatic P450s IVA1 and IVA3 catalyze the ω- and (ω-1)-hydroxylation of fatty acids and the ω-hydroxylation of prostaglandins E1 and F2a. J Lipid Res 1990; 31: 1477-1482.
75. Yamamoto S, Kusunose E, Ogita K, Kaku M, Ichihara K, Kusunose M. Isolation of cytochrome P-450 highly active in prostaglandin ω-hydroxylation from lung microsomes of rabbits treated with progesterone. J Biochem 1984; 96: 593-603.
76. Kupfer D, Jansson I, Favreau LV, Theoharides AD, Schenkman JB. Regioselective hydroxylation of prostaglandins by constitutive forms of cytochrome P-450 from rat liver: formation of a novel metabolite by a female-specific P-450. Arch Biochem Biophys 1988; 261: 186-195.
77. Sumimoto H, Kusunose E, Gotoh Y, Kusunose M, Minakami S. Leukotriene B4 ω-hydroxylase in rat liver microsomes: identification as a cytochrome P-450 that catalyzes prostaglandin A1 ω-hydroxylation, and participation of cytochrome b_5. J Biochem 1990; 108: 215-221.
78. Cantineau R, Kremers P, DeGraeve J, Gielen JE, Lambotte R. 15- and 16-hydroxylations of androgens and estrogens in the human fetal liver: a critical step in estetrol biosynthesis. J Steroid Biochem 1985; 22: 195-201.
79. Cheng K-C, Schenkman JB. Testosterone metabolism by cytochrome P-450 isozymes RLM3 and RLM5 and by microsomes. Metabolite identification. J Biol Chem 1983; 258: 11738-11744.
80. Chung B-C, Matteson KJ, Miller WL. Structure of a bovine gene for P-450c21 (steroid 21-hydroxylase) defines a novel cytochrome P-450 gene family. Proc Natl Acad Sci USA 1986; 83: 4243-4247.

81. Dannan GA, Porubek DJ, Nelson SD, Waxman DJ, Guengerich FP. 17β-Estradiol 2- and 4-hydroxylation catalyzed by rat hepatic cytochrome P-450: roles of individual forms, inductive effects, developmental patterns, and alterations by gonadectomy and hormone replacement. Endocrinology 1986; 118: 1952-1960.
82. Sheets JJ, Estabrook RW. Multiple sites of steroid hydroxylation by the liver microsomal cytochrome P-450 system: primary and secondary metabolism of androstenedione. Biochemistry 1985; 24: 6591-6597.
83. Watanabe K, Takanashi K, Imaoka S, Funae Y, Inoue K, Kamataki T, Takagi H, Yoshizawa I. Comparison of cytochrome P-450 species which catalyze the hydroxylations of the aromatic ring of estradiol and estradiol 17-sulfate. J Steroid Biochem Mol Biol 1991; 38: 737-743.
84. Waxman D, Ko A, Walsh C. Regioselectivity and stereoselectivity of androgen hydroxylations catalyzed by cytochrome P-450 isozymes purified from phenobarbital induced rat liver. J Biol Chem 1983; 258: 11937-11947.
85. Waxman D, Lapenson D, Aoyama T, Gelboin H, Gonzalez F, Korzekwa K. Steroid hormone hydroxylase specificities of eleven cDNA-expressed human cytochrome-P450s. Arch Biochem Biophys 1991; 290: 160-166.
86. Nebert DW. Proposed role of drug-metabolizing enzymes: regulation of steady state levels of the ligands that effect growth, homeostasis, differentiation, and neuroendocrine functions. Mol Endocrinol 1991; 5: 1203-1214.

Protein sequences alignments for three cytochrome P450 enzymes which are mainly reported to be responsible for catabolism of paracetamol

I) CYP1A2

1) Homo sapiens – Mus musculus

```
Score =  775 bits (2000), Expect = 0.0
Identities = 374/514 (72%), Positives = 451/514 (86%), Gaps = 3/514 (0%)


Query: 1 MALSQSVPFSATELLLASAIFCLVFWVLKGLRPRVPKGLKSPPEPWGWPLLGHVLTLGKN 60
          MA SQ +   A ELLLA+AIFCLVFW+++  R +VPKGLK+PP PWG P +GH+LT+GKN
Sbjct: 1 MAFSQYISL-APELLLATAIFCLVFWMVRASRTQVPKGLKNPPGPWGLPFIGHMLTVGKN 59

Query: 61 PHLALSRMSQRYGDVLQIRIGSTPVLVLSRLDTIRQALVRQGDDFKGRPDLYTSTLITDG 120
           PHL+L+R+SQ+YGDVLQIRIGSTPV+VLS L+TI+QALVRQGDDFKGRPDLY+ TLIT+G
Sbjct: 60 PHLSLTRLSQQYGDVLQIRIGSTPVVVLSGLNTIKQALVRQGDDFKGRPDLYSFTLITNG 119

Query: 121 QSLTFSTDSGPVWAARRRLAQNALNTFSIASDPASSSSCYLEEHVSKEAKALISRLQELM 180
           +S+TF+ DSGPVWAARRRLAQ+AL +FSIASDP S+SSCYLEEHVSKEA  L+S+LQ+ M
Sbjct: 120 KSMTFNPDSGPVWAARRRLAQDALKSFSIASDPTSASSCYLEEHVSKEANHLVSKLQKAM 179

Query: 181 AGPGHFDPYNQVVVSVANVIGAMCFGQHFPESSDEMLSLVKNTHEFVETASSGNPLDFFP 240
           A  GHF+P +QVV SVANVIGAMCFG++FP  S+EML++V N+ +FVE  +SGN +DFFP
Sbjct: 180 AEVGHFEPVSQVVESVANVIGAMCFGKNFPRKSEEMLNIVNNSKDFVENVTSGNAVDFFP 239

Query: 241 ILRYLPNPALQRFKAFNQRFLWFLQKTVQEHYQDFDKNSVRDITGALFKHSKKGPRASGN 300
           +LRYLPNPAL+RFK FN  F+ FLQKTVQEHYQDF+KNS++DIT ALFKHS +  + +G
Sbjct: 240 VLRYLPNPALKRFKTFNDNFVLFLQKTVQEHYQDFNKNSIQDITSALFKHS-ENYKDNGG 298

Query: 301 LIPQEKIVNLVNDIFGAGFDTVTTAISWSLMYLVTKPEIQRKIQKELDTVIGRERRPRLS 360
           LIP+EKIVN+VNDIFGAGFDTVTTAI+WS++ LVT P +QRKI +ELDTV+GR+R+PRLS
Sbjct: 299 LIPEEKIVNIVNDIFGAGFDTVTTAITWSILLLVTWPNVQRKIHEELDTVVGRDRQPRLS 358

Query: 361 DRPQLPYLEAFILETFRHSSFLPFTIPHSTTRDTTLNGFYIPKKCCVFVNQWQVNHDPEL 420
           DRPQLPYLEAFILE +R++SF+PFTIPHSTTRDT+LNGF+IPK+ C+++NQWQVNHD +
Sbjct: 359 DRPQLPYLEAFILEIYRYTSFVPFTIPHSTTRDTSLNGFHIPKERCIYINQWQVNHDEKQ 418

Query: 421 WEDPSEFRPERFLTADGTAINKPLSEKMMLFGMGKRRCIGEVLAKWEIFLFLAILLQQLE 480
           W+DP  FRPERFLT + +AI+K  SEK+MLFG+GKRRCIGE+ AKWE+FLFLAILLQ LE
Sbjct: 419 WKDPFVFRPERFLTNNNSAIDKTQSEKVMLFGLGKRRCIGEIPAKWEVFLFLAILLQHLE 478

Query: 481 FSVPPGVKVDLTPIYGLTMKHARCEHVQA-RRFS 513
           FSVPPGVKVDLTP YGLTMK   CEHVQA  RFS
Sbjct: 479 FSVPPGVKVDLTPNYGLTMKPGTCEHVQAWPRFS 512
```

2) Homo sapiens – Cavia porcellus

Score = 821 bits (2120), Expect = 0.0
Identities = 395/514 (76%), Positives = 456/514 (87%), Gaps = 1/514 (0%)

```
Query: 1   MALSQSVPFSATELLLASAIFCLVFWVLKGLRPRVPKGLKSPPEPWGWPLLGHVLTLGKN 60
           M LS  +PFSA ELLL + IF LV WV+K  R +VPKGLKSPP PWGWPL+GHVLTLGKN
Sbjct: 1   MPLSWLLPFSAMELLLTATIFYLVLWVVKAFRLQVPKGLKSPPGPWGWPLIGHVLTLGKN 60

Query: 61  PHLALSRMSQRYGDVLQIRIGSTPVLVLSRLDTIRQALVRQGDDFKGRPDLYTSTLITDG 120
           PHLAL+R+S RYGDVLQIRIGSTPV+VLS LDTIRQALVRQ DDFKGRPDLY+ST I+DG
Sbjct: 61  PHLALTRLSARYGDVLQIRIGSTPVVVLSGLDTIRQALVRQSDDFKGRPDLYSSTFISDG 120

Query: 121 QSLTFSTDSGPVWAARRRLAQNALNTFSIASDPASSSSCYLEEHVSKEAKALISRLQELM 180
           QS+ F+ DSGPVWAARRRLAQ+AL +FS+ASDPAS SSCYLEEHVS+EA+ L+++L +LM
Sbjct: 121 QSMIFNPDSGPVWAARRRLAQSALQSFSVASDPASVSSCYLEEHVSREAEHLVTKLLDLM 180

Query: 181 AGPGHFDPYNQVVVSVANVIGAMCFGQHFPESSDEMLSLVKNTHEFVETASSGNPLDFFP 240
           AGPG F+P +Q+V SVANVIGAMCFG++FP++S+EML +V  + EF E ASSGNP+DFFP
Sbjct: 181 AGPGCFEPSSQIVGSVANVIGAMCFGKNFPQTSEEMLQIVNTSKEFTEFASSGNPVDFFP 240

Query: 241 ILRYLPNPALQRFKAFNQRFLWFLQKTVQEHYQDFDKNSVRDITGALFKHSKKGPRASGN 300
           ILRYLPNP LQ+FK FN+RFL FLQKTVQEHYQDFDKN V+DI  ALFKHS++ P  +G+
Sbjct: 241 ILRYLPNPMLQQFKDFNKRFLQFLQKTVQEHYQDFDKNHVQDIASALFKHSEESPHVNGD 300

Query: 301 LIPQEKIVNLVNDIFGAGFDTVTTAISWSLMYLVTKPEIQRKIQKELDTVIGRERRPRLS 360
           LIP++KIVNLVNDIFGAGFDTVTTAISWSL+YLVTKPEIQ+KI KELD VIGR+R+PRL+
Sbjct: 301 LIPRKKIVNLVNDIFGAGFDTVTTAISWSLLYLVTKPEIQKKIHKELDAVIGRDRKPRLA 360

Query: 361 DRPQLPYLEAFILETFRHSSFLPFTIPHSTTRDTTLNGFYIPKKCCVFVNQWQVNHDPEL 420
           DRPQLPY+EAFILE FR+SSFLPFTIPH TTRDT LNGFYIPK  CVF+NQWQVNHDP+
Sbjct: 361 DRPQLPYMEAFILEVFRYSSFLPFTIPHCTTRDTILNGFYIPKDRCVFINQWQVNHDPKQ 420

Query: 421 WEDPSEFRPERFLTADGTAINKPLSEKMMLFGMGKRRCIGEVLAKWEIFLFLAILLQQLE 480
           WEDP EFRPERFL A+ TA++K LS+K++LFG+GKRRCIGE L +WE+FLFLAILLQQLE
Sbjct: 421 WEDPFEFRPERFLLANNTAVDKTLSDKILLFGLGKRRCIGETLGRWEVFLFLAILLQQLE 480

Query: 481 FSVPPGVKVDLTPIYGLTMKHARCEHVQAR-RFS 513
           FSVPPGVKVDLTP+YGLTMK   C+HVQAR RFS
Sbjct: 481 FSVPPGVKVDLTPVYGLTMKPPHCQHVQARPRFS 514
```

3) Homo sapiens – Rattus norvegicus

Score = 791 bits (2044), Expect = 0.0
Identities = 385/514 (74%), Positives = 455/514 (87%), Gaps = 3/514 (0%)

```
Query: 1   MALSQSVPFSATELLLASAIFCLVFWVLKGLRPRVPKGLKSPPEPWGWPLLGHVLTLGKN 60
           MA SQ +   A ELLLA+AIFCLVFWVL+G R +VPKGLKSPP PWG P +GH+LTLGKN
Sbjct: 1   MAFSQYISL-APELLLATAIFCLVFWVLRGTRTQVPKGLKSPPGPWGLPFIGHMLTLGKN 59

Query: 61  PHLALSRMSQRYGDVLQIRIGSTPVLVLSRLDTIRQALVRQGDDFKGRPDLYTSTLITDG 120
           PHL+L+++SQ+YGDVLQIRIGSTPV+VLS L+TI+QALV+QGDDFKGRPDLY+ TLIT+G
Sbjct: 60  PHLSLTKLSQQYGDVLQIRIGSTPVVVLSGLNTIKQALVKQGDDFKGRPDLYSFTLITNG 119

Query: 121 QSLTFSTDSGPVWAARRRLAQNALNTFSIASDPASSSSCYLEEHVSKEAKALISRLQELM 180
           +S+TF+ DSGPVWAARRRLAQ+AL +FSIASDP S SSCYLEEHVSKEA  LIS+ Q+LM
Sbjct: 120 KSMTFNPDSGPVWAARRRLAQDALKSFSIASDPTSVSSCYLEEHVSKEANHLISKFQKLM 179

Query: 181 AGPGHFDPYNQVVVSVANVIGAMCFGQHFPESSDEMLSLVKNTHEFVETASSGNPLDFFP 240
           A  GHF+P NQVV SVANVIGAMCFG++FP  S+EML+LVK++ +FVE  +SGN +DFFP
Sbjct: 180 AEVGHFEPVNQVVESVANVIGAMCFGKNFPRKSEEMLNLVKSSKDFVENVTSGNAVDFFP 239

Query: 241 ILRYLPNPALQRFKAFNQRFLWFLQKTVQEHYQDFDKNSVRDITGALFKHSKKGPRASGN 300
           +LRYLPNPAL+RFK FN  F+  LQKTVQEHYQDF+KNS++DITGALFKHS +  + +G
Sbjct: 240 VLRYLPNPALKRFKNFNDNFVLSLQKTVQEHYQDFNKNSIQDITGALFKHS-ENYKDNGG 298

Query: 301 LIPQEKIVNLVNDIFGAGFDTVTTAISWSLMYLVTKPEIQRKIQKELDTVIGRERRPRLS 360
           LIPQEKIVN+VNDIFGAGF+TVTTAI WS++ LVT+P++QRKI +ELDTVIGR+R+PRLS
Sbjct: 299 LIPQEKIVNIVNDIFGAGFETVTTAIFWSILLLVTEPKVQRKIHEELDTVIGRDRQPRLS 358

Query: 361 DRPQLPYLEAFILETFRHSSFLPFTIPHSTTRDTTLNGFYIPKKCCVFVNQWQVNHDPEL 420
           DRPQLPYLEAFILE +R++SF+PFTIPHSTTRDT+LNGF+IPK+CC+F+NQWQVNHD +
Sbjct: 359 DRPQLPYLEAFILEIYRYTSFVPFTIPHSTTRDTSLNGFHIPKECCIFINQWQVNHDEKQ 418

Query: 421 WEDPSEFRPERFLTADGTAINKPLSEKMMLFGMGKRRCIGEVLAKWEIFLFLAILLQQLE 480
           W+DP  FRPERFLT D TAI+K LSEK+MLFG+GKRRCIGE+ AKWE+FLFLAILL QLE
Sbjct: 419 WKDPFVFRPERFLTNDNTAIDKTLSEKVMLFGLGKRRCIGEIPAKWEVFLFLAILLHQLE 478

Query: 481 FSVPPGVKVDLTPIYGLTMKHARCEHVQA-RRFS 513
           F+VPPGVKVDLTP YGLTMK   CEHVQA  RFS
Sbjct: 479 FTVPPGVKVDLTPSYGLTMKPRTCEHVQAWPRFS 512
```

II) CYP3A4

1) Homo sapiens Sus scrofa

```
Score =  798 bits (2061), Expect = 0.0
Identities = 382/503 (75%), Positives = 439/503 (86%)

Query: 1   MALIPDLAMETWLLLAVSLVLLYLYGTHSHGLFKKLGIPGPTPLPFLGNILSYHKGFCMF 60
           M LIP  + ETW+LLA SLVLLYLYGT+SHGLFKKLGIPGP PLP+ GNIL Y KG   F
Sbjct: 1   MDLIPGFSTETWVLLATSLVLLYLYGTYSHGLFKKLGIPGPRPLPYFGNILGYRKGVDHF 60

Query: 61  DMECHKKYGKVWGFYDGQQPVLAITDPDMIKTVLVKECYSVFTNRRPFGPVGFMKSAISI 120
           D +C ++YGK+WGF+DG+QPVLAITDPDMIKTVLVKECYSVFTNRR FGP G M++A+S+
Sbjct: 61  DKKCFQQYGKMWGFFDGRQPVLAITDPDMIKTVLVKECYSVFTNRRSFGPRGAMRTALSL 120

Query: 121 AEDEEWKRLRSLLSPTFTSGKLKEMVPIIAQYGDVLVRNLRREAETGKPVTLKDVFGAYS 180
           AEDEEWKR+R+LLSPTFTSGKLKEM PII+ YGD+LV NLR+EAE GKPVT+KD+FGAYS
Sbjct: 121 AEDEEWKRIRTLLSPTFTSGKLKEMFPIISHYGDLLVSNLRKEAEKGKPVTMKDIFGAYS 180

Query: 181 MDVITSTSFGVNIDSLNNPQDPFVENTKKLLRFDFLDPFFLSITVFPFLIPILEVLNICV 240
           MDVITST+FGVN D LNNPQDPFVEN+KKLL+F F  P FLSI  FPFL PILEVLN+ +
Sbjct: 181 MDVITSTAFGVNTDFLNNPQDPFVENSKKLLKFSFFSPLFLSIIFFPFLTPILEVLNVTL 240

Query: 241 FPREVTNFLRKSVKRMKESRLEDTQKHRVDFLQLMIDSQNSKETESHKALSDLELVAQSI 300
           FP+ V NF  +S+KRMKESRL+D Q HRVDFLQLMI+SQNSKET++HK LSD ELVAQ +
Sbjct: 241 FPKSVVNFFMRSIKRMKESRLKDKQTHRVDFLQLMINSQNSKETDTHKGLSDEELVAQGV 300

Query: 301 IFIFAGYETTSSVLSFIMYELATHPDVQQKLQEEIDAVLPNKAPPTYDTVLQMEYLDMVV 360
            FIFAGYETTSS LS ++YELATHPDVQQKLQEEIDA  P+KA P+YD + QMEYLDMVV
Sbjct: 301 FFIFAGYETTSSSLSLLVYELATHPDVQQKLQEEIDATFPSKALPSYDALAQMEYLDMVV 360

Query: 361 NETLRLFPIAMRLERVCKKDVEINGMFIPKGVVVMIPSYALHRDPKYWTEPEKFLPERFS 420
           NE LRL+PIA RLERVCKKDVEI+G+ +PKG V+M+P +++HRDP+ W EPE+F PERFS
Sbjct: 361 NEILRLYPIAARLERVCKKDVEIHGVSVPKGTVMMVPVFSIHRDPELWPEPEEFRPERFS 420

Query: 421 KKNKDNIDPYIYTPFGSGPRNCIGMRFALMNMKLALIRVLQNFSFKPCKETQIPLKLSLG 480
           KKNKD+I+PY Y PFG+GPRNCIGMRFALMNMKLAL+RVLQNFSFKPCKETQ PLKLS
Sbjct: 421 KKNKDSINPYTYLPFGTGPRNCIGMRFALMNMKLALVRVLQNFSFKPCKETQTPLKLSSQ 480

Query: 481 GLLQPEKPVVLKVESRDGTVSGA 503
           GL+QPEKP++LKV  RDGTV GA
Sbjct: 481 GLIQPEKPILLKVVPRDGTVGGA 503
```

III) CYP2E1

1) Homo sapiens – Mus musculus

```
Score =  826 bits (2133), Expect = 0.0
Identities = 385/493 (78%), Positives = 445/493 (90%)

Query: 1   MSALGVTVALLVWAAFLLLVSMWRQVHSSWNLPPGPFPLPIIGNLFQLELKNIPKSFTRL 60
           M+ LG+TVALLVW A LLLVS+W+Q++ SWNLPPGPFP+P  GN+FQL+LK+IPKS T+L
Sbjct: 1   MAVLGITVALLVWIATLLLVSIWKQIYRSWNLPPGPFPIPFFGNIFQLDLKDIPKSLTKL 60

Query: 61  AQRFGPVFTLYVGSQRMVVMHGYKAVKEALLDYKDEFSGRGDLPAFHAHRDRGIIFNNGP 120
           A+RFGPVFTL++G +R+VV+HGYKAVKE LL++K+EFSGRGD+P F  ++++GIIFNNGP
Sbjct: 61  AKRFGPVFTLHLGQRRIVVLHGYKAVKEVLLNHKNEFSGRGDIPVFQEYKNKGIIFNNGP 120

Query: 121 TWKDIRRFSLTTLRNYGMGKQGNESRIQREAHFLLEALRKTQGQPFDPTFLIGCAPCNVI 180
           TWKD+RRFSL+ LR++GMGKQGNE+RIQREAHFL+E L+KT+GQPFDPTFLIGCAPCNVI
Sbjct: 121 TWKDVRRFSLSILRDWGMGKQGNEARIQREAHFLVEELKKTKGQPFDPTFLIGCAPCNVI 180

Query: 181 ADILFRKHFDYNDEKFLRLMYLFNENFHLLSTPWLQLYNNFPSFLHYLPGSHRKVIKNVA 240
           ADILF K FDY+D+K L LM LFNENF+LLSTPW+Q YN F  +L YLPGSHRKV+KNV+
Sbjct: 181 ADILFNKRFDYDDKKCLELMSLFNENFYLLSTPWIQAYNYFSDYLQYLPGSHRKVMKNVS 240

Query: 241 EVKEYVSERVKEHHQSLDPNCPRDLTDCLLVEMEKEKHSAERLYTMDGITVTVADLFFAG 300
           E+++Y   + KEH +SLD NCPRD+TDCLL+EMEKEKHS E +YTM+ I+VT+ADLFFAG
Sbjct: 241 EIRQYTLGKAKEHLKSLDINCPRDVTDCLLIEMEKEKHSQEPMYTMENISVTLADLFFAG 300

Query: 301 TETTSTTLRYGLLILMKYPEIEEKLHEEIDRVIGPSRIPAIKDRQEMPYMDAVVHEIQRF 360
           TETTSTTLRYGLLILMKYPEIEEKLHEEIDRVIGPSR PA++DR  MPYMDAVVHEIQRF
Sbjct: 301 TETTSTTLRYGLLILMKYPEIEEKLHEEIDRVIGPSRAPAVRDRMNMPYMDAVVHEIQRF 360

Query: 361 ITLVPSNLPHEATRDTIFRGYLIPKGTVVVPTLDSVLYDNQEFPDPEKFKPEHFLNENGK 420
           I LVPSNLPHEATRDT+FRGY+IPKGTVV+PTLDS+L+DN EFPDPE FKPEHFLNENGK
Sbjct: 361 INLVPSNLPHEATRDTVFRGYVIPKGTVVIPTLDSLLFDNYEFPDPETFKPEHFLNENGK 420

Query: 421 FKYSDYFKPFSTGKRVCAGEGLARMELFLLLCAILQHFNLKPLVDPKDIDLSPIHIGFGC 480
           FKYSDYFK FS GKRVC GEGLARMELFLLL AILQHFNLK LVDPKDIDLSP+ IGFG
Sbjct: 421 FKYSDYFKAFSAGKRVCVGEGLARMELFLLLSAILQHFNLKSLVDPKDIDLSPVTIGFGS 480

Query: 481 IPPRYKLCVIPRS 493
           IP  +KLCVIPRS
Sbjct: 481 IPREFKLCVIPRS 493
```

2) Homo sapiens - Rattus norvegicus

```
Score =  843 bits (2179), Expect = 0.0
Identities = 388/493 (78%), Positives = 457/493 (91%)


Query: 1   MSALGVTVALLVWAAFLLLVSMWRQVHSSWNLPPGPFPLPIIGNLFQLELKNIPKSFTRL 60
           M+ LG+T+ALLVW A LL++S+W+++++SWNLPPGPFPLPI+GN+FQL+LK+IPKSFT+L
Sbjct: 1   MAVLGITIALLVWVATLLVISIWKKIYNSWNLPPGPFPLPILGNIFQLDLKDIPKSFTKL 60

Query: 61  AQRFGPVFTLYVGSQRMVVMHGYKAVKEALLDYKDEFSGRGDLPAFHAHRDRGIIFNNGP 120
           A+RFGPVFTL++GS+R+VV+HGYKAVKE LL++K+EFSGRGD+P F  ++++GIIFNNGP
Sbjct: 61  AKRFGPVFTLHLGSRRIVVLHGYKAVKEVLLNHKNEFSGRGDIPVFQEYKNKGIIFNNGP 120

Query: 121 TWKDIRRFSLTTLRNYGMGKQGNESRIQREAHFLLEALRKTQGQPFDPTFLIGCAPCNVI 180
           TWKD+RRFSL+ LR++GMGKQGNE+RIQREA FL+E L+KT+GQPFDPTFLIGCAPCNVI
Sbjct: 121 TWKDVRRFSLSILRDWGMGKQGNEARIQREAQFLVEELKKTKGQPFDPTFLIGCAPCNVI 180

Query: 181 ADILFRKHFDYNDEKFLRLMYLFNENFHLLSTPWLQLYNNFPSFLHYLPGSHRKVIKNVA 240
           ADILF K FDYND+K LRLM LFNENF+LLSTPW+QLYNNF  +L YLPGSHRK++KNV+
Sbjct: 181 ADILFNKRFDYNDKKCLRLMSLFNENFYLLSTPWIQLYNNFADYLRYLPGSHRKIMKNVS 240

Query: 241 EVKEYVSERVKEHHQSLDPNCPRDLTDCLLVEMEKEKHSAERLYTMDGITVTVADLFFAG 300
           E+K+Y  E+ KEH QSLD NC RD+TDCLL+EMEKEKHS E +YTM+ ++VT+ADLFFAG
Sbjct: 241 EIKQYTLEKAKEHLQSLDINCARDVTDCLLIEMEKEKHSQEPMYTMENVSVTLADLFFAG 300

Query: 301 TETTSTTLRYGLLILMKYPEIEEKLHEEIDRVIGPSRIPAIKDRQEMPYMDAVVHEIQRF 360
           TETTSTTLRYGLLILMKYPEIEEKLHEEIDRVIGPSR+PA++DR +MPYMDAVVHEIQRF
Sbjct: 301 TETTSTTLRYGLLILMKYPEIEEKLHEEIDRVIGPSRVPAVRDRLDMPYMDAVVHEIQRF 360

Query: 361 ITLVPSNLPHEATRDTIFRGYLIPKGTVVVPTLDSVLYDNQEFPDPEKFKPEHFLNENGK 420
           I LVPSNLPHEATRDT+F+GY+IPKGTVV+PTLDS+LYD+ EFPDPEKFKPEHFLNENGK
Sbjct: 361 INLVPSNLPHEATRDTVFQGYVIPKGTVVIPTLDSLLYDSHEFPDPEKFKPEHFLNENGK 420

Query: 421 FKYSDYFKPFSTGKRVCAGEGLARMELFLLLCAILQHFNLKPLVDPKDIDLSPIHIGFGC 480
           FKYSDYFK FS GKRVC GEGLARMELFLLL AILQHFNLK LVDPKDIDLSP+ +GFG
Sbjct: 421 FKYSDYFKAFSAGKRVCVGEGLARMELFLLLSAILQHFNLKSLVDPKDIDLSPVTVGFGS 480

Query: 481 IPPRYKLCVIPRS 493
           IPP++KLCVIPRS
Sbjct: 481 IPPQFKLCVIPRS 493
```

3) Homo sapiens – Canis familiaris (dog)

```
Score =  807 bits (2085), Expect = 0.0
Identities = 381/493 (77%), Positives = 435/493 (87%)

Query: 1   MSALGVTVALLVWAAFLLLVSMWRQVHSSWNLPPGPFPLPIIGNLFQLELKNIPKSFTRL 60
           M+ALG+TVALLVW A L+L+S+W+Q++S W LPPGPFPLPIIGN+ Q+++KN+PKS  +L
Sbjct: 1   MAALGITVALLVWMATLMLISIWKQIYSRWKLPPGPFPLPIIGNILQVDIKNVPKSLAKL 60

Query: 61  AQRFGPVFTLYVGSQRMVVMHGYKAVKEALLDYKDEFSGRGDLPAFHAHRDRGIIFNNGP 120
           A+++GPVFTLY+GSQR VV+HGYKAVKE LLD+K++ SGRG++ AF +H+DRGI FNNGP
Sbjct: 61  AEQYGPVFTLYLGSQRTVVLHGYKAVKEVLLDHKNDLSGRGEVFAFQSHKDRGITFNNGP 120

Query: 121 TWKDIRRFSLTTLRNYGMGKQGNESRIQREAHFLLEALRKTQGQPFDPTFLIGCAPCNVI 180
            WKD RR SL+TLR+YGMGK+GNE RIQRE  FLLEALR T+GQPFDPTFL+G AP NVI
Sbjct: 121 GWKDTRRLSLSTLRDYGMGKRGNEERIQREIPFLLEALRGTRGQPFDPTFLLGFAPFNVI 180

Query: 181 ADILFRKHFDYNDEKFLRLMYLFNENFHLLSTPWLQLYNNFPSFLHYLPGSHRKVIKNVA 240
           ADILF KHFDY+D+  LR+  LFNENFHLLST WLQLYN FPS+LHYLPGSHRKV++NVA
Sbjct: 181 ADILFHKHFDYSDQTGLRIQKLFNENFHLLSTGWLQLYNIFPSYLHYLPGSHRKVLRNVA 240

Query: 241 EVKEYVSERVKEHHQSLDPNCPRDLTDCLLVEMEKEKHSAERLYTMDGITVTVADLFFAG 300
           E+K+Y  ERVKEH +SLDP C RD TDCLL E++KE++  E  YT+D I VTVADLFFAG
Sbjct: 241 ELKDYSLERVKEHQESLDPTCSRDFTDCLLQELQKERYGTEPWYTLDNIAVTVADLFFAG 300

Query: 301 TETTSTTLRYGLLILMKYPEIEEKLHEEIDRVIGPSRIPAIKDRQEMPYMDAVVHEIQRF 360
           TETTSTTLRYGLLILMKYPE+EEKLHEEIDRVIGPSR+PAIKDR EMPYMDAVVHEIQRF
Sbjct: 301 TETTSTTLRYGLLILMKYPEVEEKLHEEIDRVIGPSRVPAIKDRLEMPYMDAVVHEIQRF 360

Query: 361 ITLVPSNLPHEATRDTIFRGYLIPKGTVVVPTLDSVLYDNQEFPDPEKFKPEHFLNENGK 420
           I L+PSNLPH A +DT+FRGY+IPKGTVV+PTLDSVL+D QEFPDPEKFKPEHFLNENGK
Sbjct: 361 IDLLPSNLPHVANQDTMFRGYVIPKGTVVIPTLDSVLFDKQEFPDPEKFKPEHFLNENGK 420

Query: 421 FKYSDYFKPFSTGKRVCAGEGLARMELFLLLCAILQHFNLKPLVDPKDIDLSPIHIGFGC 480
           FKYSDYFK FS GKRVC GEGLARMELFL L AILQHFNLK LVDPKDIDLSP  IGF
Sbjct: 421 FKYSDYFKAFSAGKRVCVGEGLARMELFLFLSAILQHFNLKSLVDPKDIDLSPCTIGFAK 480

Query: 481 IPPRYKLCVIPRS 493
           IPP YKLCV+PRS
Sbjct: 481 IPPHYKLCVVPRS 493
```

4) Homo sapiens – Sus scrofa (pig)

```
Score =  843 bits (2178), Expect = 0.0
Identities = 392/493 (79%), Positives = 448/493 (90%)


Query: 1   MSALGVTVALLVWAAFLLLVSMWRQVHSSWNLPPGPFPLPIIGNLFQLELKNIPKSFTRL 60
           M+ALG+TVALLVW   LLL+S+W+ +HSSW LPPGPFPLPI+GN+FQL+LKNIPKSFT L
Sbjct: 1   MTALGITVALLVWLVTLLLISIWKHIHSSWKLPPGPFPLPIVGNIFQLDLKNIPKSFTML 60

Query: 61  AQRFGPVFTLYVGSQRMVVMHGYKAVKEALLDYKDEFSGRGDLPAFHAHRDRGIIFNNGP 120
           A+R+GPVFT+Y+GS+R+VV+HGYKAVKE LL YK+EFSGRG++P F  H+D+G+IFNNGP
Sbjct: 61  AERYGPVFTVYLGSRRIVVLHGYKAVKEVLLHYKNEFSGRGEIPTFQVHKDKGVIFNNGP 120

Query: 121 TWKDIRRFSLTTLRNYGMGKQGNESRIQREAHFLLEALRKTQGQPFDPTFLIGCAPCNVI 180
           TW+D RRFSLTTLR++GMGKQGNE RIQREAHFLLEALRKT GQPFDPTFLIGCAPCNVI
Sbjct: 121 TWRDTRRFSLTTLRDFGMGKQGNEQRIQREAHFLLEALRKTHGQPFDPTFLIGCAPCNVI 180

Query: 181 ADILFRKHFDYNDEKFLRLMYLFNENFHLLSTPWLQLYNNFPSFLHYLPGSHRKVIKNVA 240
           +DILFR+HFDYND+  LRLM +FNENF+LLST W+QLYNNF  +L YLPGSHRK++KN++
Sbjct: 181 SDILFRQHFDYNDKTCLRLMSMFNENFYLLSTGWIQLYNNFSGYLRYLPGSHRKLMKNIS 240

Query: 241 EVKEYVSERVKEHHQSLDPNCPRDLTDCLLVEMEKEKHSAERLYTMDGITVTVADLFFAG 300
           E+K+Y  ERVK+H  SL+P+CPRD TD LL+EMEKEK+SAE +YT+D I VTVAD+FFAG
Sbjct: 241 EIKDYALERVKDHRDSLEPSCPRDFTDTLLMEMEKEKYSAEPIYTLDNIAVTVADMFFAG 300

Query: 301 TETTSTTLRYGLLILMKYPEIEEKLHEEIDRVIGPSRIPAIKDRQEMPYMDAVVHEIQRF 360
           TETTSTTLRYGLLILMKYPE+EEKLHEEIDRVIGP+RIPAIKDR  MPY+DAVVHEIQRF
Sbjct: 301 TETTSTTLRYGLLILMKYPEVEEKLHEEIDRVIGPNRIPAIKDRLVMPYLDAVVHEIQRF 360

Query: 361 ITLVPSNLPHEATRDTIFRGYLIPKGTVVVPTLDSVLYDNQEFPDPEKFKPEHFLNENGK 420
           I L+PSNLPHEATRDT FR Y+IPKGTVV+PTLDSVLYD+QEFP+PEKFKPEHFLNENGK
Sbjct: 361 IDLIPSNLPHEATRDTDFRDYIIPKGTVVIPTLDSVLYDSQEFPEPEKFKPEHFLNENGK 420

Query: 421 FKYSDYFKPFSTGKRVCAGEGLARMELFLLLCAILQHFNLKPLVDPKDIDLSPIHIGFGC 480
           FKYSD+FK FS GKRVC GEGLARMELFL + AILQHFNLK LVDPKDIDLSPI IGF
Sbjct: 421 FKYSDHFKAFSAGKRVCVGEGLARMELFLFMAAILQHFNLKSLVDPKDIDLSPIAIGFAK 480

Query: 481 IPPRYKLCVIPRS 493
           IPP YKLCVIPRS
Sbjct: 481 IPPHYKLCVIPRS 493
```

4) Homo sapiens - Mesocricetus auratus (hamster)

```
Score =  836 bits (2160), Expect = 0.0
Identities = 385/493 (78%), Positives = 450/493 (91%)


Query: 1   MSALGVTVALLVWAAFLLLVSMWRQVHSSWNLPPGPFPLPIIGNLFQLELKNIPKSFTRL 60
           M+  GVT+ALLVW A LL+VS+W+Q++SSWNLPPGPFPLPI+GN+FQL+LKNIPKS T+L
Sbjct: 1   MAVFGVTIALLVWVATLLIVSIWKQIYSSWNLPPGPFPLPILGNIFQLDLKNIPKSLTKL 60

Query: 61  AQRFGPVFTLYVGSQRMVVMHGYKAVKEALLDYKDEFSGRGDLPAFHAHRDRGIIFNNGP 120
           A+RFGPVFTL++GS+R+VV+HGYKAVKE LL++K+EFSGRGD+P F  + ++GIIFNNGP
Sbjct: 61  AERFGPVFTLHLGSKRIVVLHGYKAVKEVLLNHKNEFSGRGDIPVFQEYMNKGIIFNNGP 120

Query: 121 TWKDIRRFSLTTLRNYGMGKQGNESRIQREAHFLLEALRKTQGQPFDPTFLIGCAPCNVI 180
           TWKD+RRFSL+ LR+YGMGKQGNE+RIQREAHFL+E L+KT GQPFDPTFL+GCAP NVI
Sbjct: 121 TWKDVRRFSLSILRDYGMGKQGNEARIQREAHFLMEELKKTNGQPFDPTFLVGCAPFNVI 180

Query: 181 ADILFRKHFDYNDEKFLRLMYLFNENFHLLSTPWLQLYNNFPSFLHYLPGSHRKVIKNVA 240
           +DILF K FDYND+  LRLM LFNENF+LLSTPW+Q YNNF ++L YLPGSHRK++KN +
Sbjct: 181 SDILFHKRFDYNDKTCLRLMSLFNENFYLLSTPWIQAYNNFENYLRYLPGSHRKIMKNAS 240

Query: 241 EVKEYVSERVKEHHQSLDPNCPRDLTDCLLVEMEKEKHSAERLYTMDGITVTVADLFFAG 300
           E+++Y   + KEH QSLD +CPRD+TDCLL+EMEKEK S E +YTM+ I+VT+ADLFFAG
Sbjct: 241 EIRQYTLAKAKEHLQSLDSSCPRDVTDCLLIEMEKEKDSQEPMYTMENISVTLADLFFAG 300

Query: 301 TETTSTTLRYGLLILMKYPEIEEKLHEEIDRVIGPSRIPAIKDRQEMPYMDAVVHEIQRF 360
           TETTSTTLRYGLLILMKYPE+EEKLHEEIDRVIGPSR+P  KDR EMPYMDAVVHEIQRF
Sbjct: 301 TETTSTTLRYGLLILMKYPEVEEKLHEEIDRVIGPSRVPVFKDRLEMPYMDAVVHEIQRF 360

Query: 361 ITLVPSNLPHEATRDTIFRGYLIPKGTVVVPTLDSVLYDNQEFPDPEKFKPEHFLNENGK 420
           I+L+PSNLPHEATRDT+FRGY+IPKGTVV+PTLDS+LYD+QEFPDPEKFKPEHFLNENGK
Sbjct: 361 ISLIPSNLPHEATRDTMFRGYVIPKGTVVIPTLDSLLYDSQEFPDPEKFKPEHFLNENGK 420


Query: 421 FKYSDYFKPFSTGKRVCAGEGLARMELFLLLCAILQHFNLKPLVDPKDIDLSPIHIGFGC 480
           FKYSD+FK FS GKRVC GEGLARMELFLLL AILQHFNLK LVDPKDIDL+P+ IGFGC
Sbjct: 421 FKYSDHFKAFSAGKRVCVGEGLARMELFLLLTAILQHFNLKSLVDPKDIDLNPVTIGFGC 480

Query: 481 IPPRYKLCVIPRS 493
           +PP +KLCVIPRS
Sbjct: 481 VPPEFKLCVIPRS 493
```

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application No.	: 09/869,696	Confirmation No.	: 8554
Applicant	: Donald DAVIES		
Filed	: October 31, 2001		
TC/A.U.	: 1636		
Examiner	: Maria MARVICH		
Docket No.	: 010331.49927US		
Customer No.	: 23911		
Title	: P450/ACETAMINOPHEN GDEPT FOR CANCER TREATMENT		

DECLARATION

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Donald Davies, a Citizen of the United Kingdom, residing at 7 Mynchen Close, Beaconsfield, Buckinghamshire, United Kingdom, HP9 2AU, do hereby solemnly and sincerely declare, THAT

1. I graduated with honours in Chemistry from the University of Wales and I have a PhD in Biochemistry from the University of London..

2. I am a the Director of Research and Development at ML Laboratories plc and Professor of Biochemical Pharmacology at Imperial College London..

3. I am a Fellow of the Royal College of Physicians and the Royal College of Pathologists , London.

4. I am the sole named inventor for the above-identified United States patent application and am well aware of its prosecution history.

5. Data in the specification (page 21, first paragraph) show that transient transfection of COS cells using LiPofectAMINE (LPA) (Gibco BRL UK) with a vector carrying a P450 enzyme (CYP1A2) under the control of a promoter which shows enhanced expression in tumor cells is capable of sensitizing the cells to be killed by therapeutically relevant concentrations of acetaminophen.

6. Data in the specification also show that tumor cells stably expressing a P450 enzyme are sensitive to killing by acetaminophen within a six (6) hour time period and also generate significant NABQI that kills surrounding by-stander cells that do not express the enzyme (Figure 4 and Table 1 of the Specification).

7. The following summarizes results of experiments which were conducted by me personally or under my control or supervision.

8. The following experiments extend the data in the specification using clinically relevant E1 deleted adenoviral vectors to deliver and transiently express both human and murine CYP1A2 (mCYP1A2) enzymes.

9. The Following experiments further demonstrate that mCYP1A2/acetaminophen GDEPT is effective *in vivo*, using a mouse HepG2 xenograft model.

Summary of Data

10. **Summary of *in vivo* data**: El-deleted replication-defective adenoviruses expressing human CYP1A2 (Ad.hCYP1A2) and murine CYP1A2 (Ad.mCYP1A2) were prepared. HepG2 xenografts of Balb/C mice were i.t. injected with Ad.mCYP1A2, followed by i.p. administration of acetaminophen. Overall, around 50% of tumors either regressed or did not increase in size. It is concluded that CYP1A2-mediated conversion of acetaminophen to NABQI is an effective and safe gene-directed enzyme prodrug therapy (GDEPT) system for cancer treatment.

11. Summary of experiments using various cell lines: Infection of cell lines derived from a range of tumor types (hepatocellular carcinoma, prostate and colon) with either Ad.hCYP1A2 or Ad.mCYP1A2 sensitised them to killing by acetaminophen in a dose dependent manner.

12. Modulation of cellular glutathione (GSH) levels using buthionine sulphoximine (BSO) improved cell-killing efficacy.

13. Ad.mCYP1A2 showed improved killing compared to Ad.hCYP1A2, a profile consistent with known species-specific acetaminophen toxicity.

14. Selective inhibition of hCYP1A2 was demonstrated using the non-competitive hCYP1A2 inhibitor furafylline, suggesting that the liver of the patient can be protected by selective inhibition of endogenous hCYP1A2.

Materials and Methods

15. ***Tissue Culture.*** All cell lines with exception of B16F1 were obtained from ATCC (Manassas, VA, USA). HepG2, CMT93, HCT116, SW480, Hepa1-6, SK-OV-3 and V79 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and antibiotics: 50 U/ml penicillin and 50 µg/ml streptomycin. MCF-7 and DU-145 cells were in Eagle's Minimum Essential Medium (EMEM) medium, 10% FCS, 2 mM L-glutamine and 1% Non-Essential Amino Acids (NEAA, Sigma-Aldrich, Poole, UK) and antibiotics as mentioned above. 4T1 were cultured in RPMI containing 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, 10% FCS and antibiotics as mentioned above. B16F1 (NCI Tumour Bank, Frederick, MD, USA) were cultured in EMEM supplemented with 10% FCS, 1% NEAA and 25 U/ml penicillin and 25 µg/ml streptomycin. PER.C6 cells were obtained from Crucell (Leiden, The Netherlands). 911 cells were kindly provided by Prof. L. S. Young (Cancer Research UK Institute for Cancer Studies, University of Birmingham, Birmingham, UK) and were cultured in DMEM containing 10% FCS, 1 mM L-glutamine, 10 mM $MgCl_2$ and antibiotics.

16. ***Clonings and adenoviral vector construction.*** Human (hCYP1A2), hepG2 (hepCYP1A2) and murine (mCYP1A2) enzymes were cloned into recombinant adenoviral vectors following amplification of each gene by RT-PCR.

hCYP1A2. Two µg of total liver RNA (Stratagene, Amsterdam, The Netherlands) was amplified by RT-PCR after annealing oligonucleotide dT_{15} (25 µg/ml) (Promega, Madison, WI, USA) for cDNA synthesis in 1st strand synthesis buffer (Invitrogen, Groeningen, The Netherlands), 10 mM Dithiothreitol (DTT), 40 U RNAsin (Promega), 0.5 mM dNTPs (Promega) and 200 units of MMLV RT enzyme (Invitrogen) at 37°C for 60 min. Two µl of cDNA mix was used as a template in a PCR reaction using Vent DNA polymerase as recommended by the supplier (NEB, Beverly, MA, USA) with oligos CYP1N (5'-agtccaagcttgccgccagccatgg cattgtcccagtctg ttccc-3') and CYP3'end (5'-tgaggatcctcaattgat ggagaagcg-3') both at 25 µM. Sequence analysis of the product identified the need to replace amino acid 511R with 511L. This was achieved by performing an additional PCR on the amplified product as a template using oligos CYP1N and CYP3'rep (5'-tgaggatcctcaattgatggagaagcgcagccgcgcctg-3'). Following sequence confirmation, restriction digestion with *Hin*dIII and *Bam*HI enabled the fragment to be cloned under the control of the CMV promoter into

the expression vector pTX0374 . [Djeha et al, 2001, Molecular Therapy 3:233-240].

hepCYP1A2. To confirm the lack of reported functional activity of endogenous HepG2 CYP1A2, 2 μg of HepG2 cellular RNA prepared using Trizol (Invitrogen), according to the manufacturers instructions, was used in an RT-PCR reaction, as described above, with primers CYP1N and CYP3'end. Sequence analysis of hepCYP1A2 identified two amino acid changes relative to the human sequence, 84L and 269H. This gene was also cloned downstream from the CMV promoter as described above.

mCYP1A2. Murine CYP1A2 was amplified by RT-PCR using oligo dT primed total RNA (2 μg) from 3-Methylcholanthrene (Sigma-Aldrich) treated Hepa1c1c7 cells (10 uM, 24 h) using oligos mCYP1 (5'-tcaaagcttgccgccagccatggcgttctcccagtac -3') and mCYP2 (5'-tgaggatcctcacttggaaaagcgtggcc -3'). The product was digested with *Nco*I and *Bam*HI, and cloned into the expression vector pTX0460. The CMV promoter in this plasmid was replaced by first isolating a *Xho*I to *Nco*I sub-fragment which was cloned into an additional temporary vector (pTX0377). *Hin*dIII digestion of the sub-cloned fragment enabled the introduction of the CMV promoter. Cloning back the CMV promoter as *Xho*I-*Nco*I fragment upstream of the mCYP1A2 gene in pTX0460 resulted in the expression plasmid pTX0468 containing the complete expression cassette. All CYP expression cassettes contained the human β-globin gene IVSII sequence as intron and poly (A) signal.

Generation of recombinant adenovirus vectors expressing human, hepG2 and murine CYP1A2 was achieved by cloning the *Spe*I fragment from each expression construct into the transfer vector pPS1128, respectively. Recombination of the respective pPS1128 plasmids was performed with the E3-deleted adenoviral vector backbone plasmid pPS1160 in PERC6 cells to generate the replication deficient viruses Ad.hCYP1A2, Ad.mCYP1A2 and Ad.hepCYP1A2, respectively. The viruses were scaled-up, double CsCl-purified and virus titers were determined by BCA assay (Pierce, Helsingborg, Sweden) (particles/ml) and standard plaque assay (pfu/ml) on 911 cells as described in [Djeha et al, 2001, Molecular Therapy 3:233-240]

17. ***In vitro cell viability assay.*** Infection of cells with adenoviral vectors expressing CYP1A2 enzymes at a particular multiplicity of infection (MOI) was performed in suspension at 1.6 x 10^6 cells/ml in DMEM + 1% serum for 90 min at 37°C with periodic agitation to reduce cell clumping. Medium (1% FCS) was added to the infected cells which were then plated out at 0.8 x10^6 cells/ml in 100 μl amounts into a 96-well plates. Cells were incubated at 37°C in a 5% CO_2 and humidified atmosphere to allow transgene expression. The addition of acetaminophen prodrug dissolved in serum free DMEM was performed, along with the addition of buthionine sulphoximine (BSO) or

furafylline (both Sigma-Aldrich, if appropriate, to generate the desired final concentration. To quantitate cell killing, 30-40 µl of CellTiter 96 Aqueous One Solution Cell Proliferation Assay MTS substrate (Promega) was added to each well and cell viability was calculated by reading the OD at 450 nm. Percentage viability was calculated relative to uninfected controls, all readings were corrected for background absorbance.

18. ***Animal studies.*** Using HepG2 xenografts as a model of hepatocellular carcinoma, xenografts were inoculated into Balb/C mice and allowed to develop to palpable size (about 30-70 mm^3). Tumors were injected with Ad.mCYP1A2 virus using equal pfu of each virus by a single direct injection using a 27 gauge needle and a U-100 insulin syringe (Terumo, Leuven, Belgium). Two days later, acetaminophen, the prodrug, dissolved in PBS, was delivered as a single bolus i.p. injection at 300 mg/kg, a dose previously established by experiment and consistent with literature values]. Tumor progression was monitored daily and measured using Vernier calipers to estimate tumor size, expressed as a surface area determined by multiplying the longest diameter by the greatest perpendicular diameter (length x width = mm^2). Animals having a tumor xenograft of greater than 140 mm^2, or experienced a weight loss of >15% or signs of immediate ulceration were humanely sacrificed in accordance with UK government regulations. Routine follow up of the animals was performed for 50 days.

Results

19. **Expression of human CYP1A2 sensitizes HepG2 cells to acetaminophen toxicity.** To determine whether CYP1A2 expression followed by exposure of cells to acetaminophen would generate sufficient NABQI to induce cell killing, E1 deleted recombinant adenoviruses were constructed expressing CYP1A2 enzymes. Human CYP1A2 was amplified from total liver RNA (Stratagene) by RT-PCR, the sequence was identical to that published by Jaiswal et al. [1986, Nucleic Acid Research 14:6773-6774], except that, surprisingly, an additional amino acid was encoded at position 511 (ARRRF instead of ARRF). Repair of this clone to generate the ARRF sequence resulted in no detectable protein expression by Western blotting, despite an abundance of mRNA as visualised by Northern analysis (data not shown). Comparison with alternative hCYP1A2 sequences identified ARLRF as the most common sequence around this position. When 511R in the original sequence (ARRRF) was replaced by 511L (ARLRF), protein expression and EROD activity of hCYP1A2 was detected (data not shown). This sequence was used to generate adenovirus Ad.hCYP1A2 expressing human CYP1A2 from the human CMV IE enhancer/promoter. The integrity of Ad.hCYP1A2 was confirmed by restriction digestion and sequencing (data not shown).

HepG2 cells were infected with Ad.hCYP1A2 at a range of MOI (0 to 400), transgene expression was allowed for 24 h and was followed by an overnight exposure to acetaminophen prodrug. Cell killing was observed for HepG2 cells infected with Ad.hCYP1A2 in an acetaminophen dose dependent manner (Fig. 1). Cytotoxicity was dependant on hCYP1A2 expression as no killing was observed with a control virus expressing β-galactosidase (Ad.CMV-LacZ) at an identical MOI (data not shown). To determine whether any contribution to acetaminophen mediated toxicity was made from endogenous CYP1A2 activity inherent in the HepG2 cell line, amplification of CYP1A2 from HepG2 total RNA, cloning, sequencing and adenoviral expression analysis showed no detectable protein by Western blotting and no EROD activity (data not shown). Interestingly, the C-terminal ARLRF sequence was present in hepCYP1A2 along with two other coding changes (84L and 269H), suggesting these polymorphisms influenced expression. Furthermore, no significant reduction in viability was detected in uninfected HepG2 cells at each acetaminophen concentration (Fig 1), confirming the lack of a functional CYP1A2 in HepG2 cells and excluding the potential contribution of other HepG2 CYP450 enzymes to the cytotoxic effect.

20. **Modulation of cellular glutathione (GSH) improves efficacy of NABQI mediated killing** To enhance the observed cytotoxic activity of NABQI due to exposure of hCYP1A2 expressing cells to acetaminophen, modulation of cellular glutathione was investigated using buthionine sulphoximine (BSO). BSO inhibits gamma glutamyl synthase, the rate limiting enzyme for GSH biosynthesis, resulting in lowered cellular GSH reserves and a reduced capacity for NABQI inactivation by conjugation to GSH.

Improved cell killing was observed when BSO (250 μM) was included during exposure of Ad.hCYP1A2 transduced HepG2 cells to acetaminophen to inhibit de novo GSH synthesis (Fig. 2, left panel; compare to Fig. 1). In addition, pre-treatment of cells with BSO for 24 hours prior to prodrug addition further improved potency, presumably due to a greater reduction in the protective cellular GSH reserve (Fig.2, right panel). There was no evidence of reduced viability of cells due to BSO alone at the concentrations used. Therefore, GSH modulation with BSO enabled improved efficacy for Ad.hCYP1A2 at lower MOI and at lower acetaminophen concentration.

21. **CYP1A2/acetaminophen GDEPT is effective against a range of tumour cell lines** To evaluate the susceptibility of a range of cell types, including those from non-liver derived cell lines, NABQI mediated cytotoxicity was measured in a panel of cell lines (Table 1). Comparison of relative susceptibility was complicated by (i) differences in adenovirus transducibility, (ii) level of cellular GSH and, most importantly, (iii) expression of cytochrome P450 reductase. Consistent with data reported earlier [Thatcher et al, 2000, Cancer Gene Therapy 7:521-525], P450 reductase was found to be essential for NABQI-induced killing, although the minimum level of reductase required was not

established. Moreover, NABQI-mediated killing was not dependent on the p53 status of the cell as we were able to kill wild-type-(e.g. HepG2), mutant-(e.g. SW480) and null-p53 (e.g. 4T1) cell lines. We conclude, for effective CYP1A2/acetaminophen mediated killing, potential tumour targets need to express reasonable amounts of P450 reductase and have a low level of endogenous GSH, beside being transducible by adenovirus.

22. **Expression of murine CYP1A2 improves efficacy of acetaminophen mediated cell killing** Mice have an increased susceptibility to acetaminophen toxicity *in vivo*, due to enhanced activity of murine CYP1A2 [Tee et al, 1987, Biochemical Pharmacology 36:1041-1052] for NABQI formation. Therefore to further improve CYP-mediated killing, the relative efficacy of human and murine CYP1A2 GDEPT was compared *in vitro*. Murine CYP1A2 was derived by RT-PCR from Hepa 1c1c7 total cellular RNA, sequenced then cloned into the same CMV driven adenovirus vector backbone used for Ad.hCYP1A2 to produce Ad.mCYP1A2. The expression of mCYP1A2 after infections was confirmed by Western blotting (data not shown).

CMT93 cells, which showed the best susceptibility to CYP1A2/acetaminophen mediated killing (Table 1), were infected with an equal MOI of Ad.hCYP1A2 and Ad.mCYP1A2, respectively. Sensitivity of infected cells to acetaminophen was compared in the presence of BSO (Fig. 3). CMT93 cells infected with Ad.mCYP1A2 clearly showed superior killing sensitivity at all prodrug concentrations compared to Ad.hCYP1A2 infected cells (Fig. 3A). Similar results were observed in the cell lines HepG2 and Hepa 1-6 (data not shown).

In an attempt to minimize issues of relative virus titer and purity, the hCYP1A2 and mCYP1A2 comparison was repeated using newly prepared and titred virus. In this experiment, cell viability of CMT93 cells infected with Ad.hCYP1A2 and Ad.mCYP1A2 was measured at a single acetaminophen concentration across a range of MOIs to define a dose response curve (Fig. 3B). From this analysis, the increased activity of the Ad.mCYP1A2 virus was apparent by a lower MOI needed to achieve a 50% reduced cell viability along with a steeper slope of the curve. It is estimated that Ad.mCYP1A2 mediated killing was around 2-3-fold more effective than with the Ad.hCYP1A2 virus, a consistent figure observed in two additional independent experiments.

23. **Selective inhibition of human CYP1A2 using furafylline** For a GDEPT approach *in vivo*, it would be advantageous to capitalize on the increased efficacy of the mCYP1A2 enzyme in combination with maximum tolerable acetaminophen prodrug. Therefore, a strategy to protect the host liver from endogenous cytochrome P450 mediated NABQI generation may be required. This approach may effectively increase the therapeutic index for acetaminophen.

Furafylline has been shown to be a potent non-competitive inhibitor of hCYP1A2 enzyme [Sesardic et al, 1990, British Journal of Clinical Pharmacology 29:651-663]. To investigate selective inhibition of hCYP1A2, HepG2 cells were infected at a single MOI of 200 with Ad.hCYP1A2 and Ad.mCYP1A2 and then incubated for 24 h to allow transgene expression. Furafylline (0-20 μM) was added, incubated for 60 min, followed by the addition of 5 mM acetaminophen (in the presence of 250 μM BSO) for 16 h. Cell viability was determined using the MTS substrate assay kit (Fig. 4). With increasing furafylline concentration, Ad.hCYP1A2 mediated cell killing was selectively inhibited compared to Ad.mCYP1A2. Furafylline at concentrations greater than 1.0 μM completely inhibited the human enzyme, whereby mCYP1A2-mediated cytotoxicity was inhibited at furafylline concentrations only exceeding 5 μM. The approximate IC50 for furafylline was 0.1 μM for hCYP1A2 and at least 50-fold greater for mCYP1A2 (approx. 5 μM), indicating that, in principle, selective inhibition of endogenous hCYP1A2 was possible at furafylline concentrations that do not impact mCYP1A2-mediated killing.

24. **Short term exposure to acetaminophen generated sufficient NABQI to induce cell killing** The time required for acetaminophen exposure to generate sufficient NABQI to reduce cell viability was investigated. This is of clear clinical significance as the plasma peak levels of acetaminophen in humans are reached at about 30-60 min after oral or i.v. application and the half-time is about 2 h [Lit]. HepG2 cells were infected with Ad.hCYP1A2 and allowed to express hCYP1A2 for 24 h. The cells were then incubated in 5 mM acetaminophen from 1 to 24 h prior to the addition of 2 μM furafylline to inhibit any further hCYP1A2 activity. Cell viability was determined 24 h later at staggered time points to ensure that the time for cell viability determination following acetaminophen exposure was constant (Fig. 5).

There was a clear reduction in cell viability corresponding to the level of CYP1A2 expression and the time for NABQI generation. Control cells infected with an Ad.CMV-LacZ virus did not exhibit any reduction in viability under these conditions (data not shown). For Ad.hCYP1A2 at an MOI of 200, cell viability was reduced to 20% as a consequence of NABQI formation by hCYP1A2 for around 6 hours. Therefore CYP1A2 expression and a short term exposure to acetaminophen generated sufficient NABQI to be highly effective. Due to the enhanced activity of mCYP1A2, the exposure time to acetaminophen may be reduced yet further which is favorable for the clinical situation.

26. ***In vivo* anti-tumor efficacy** To demonstrate efficacy of the CYP1A2/acetaminophen system *in vivo*, HepG2 xenografts were prepared in Balb/C nude mice. Tumours were allowed to develop to 30-70 mm^3 size and were then injected with a single dose of 1.0×10^{10} Ad.mCYP1A2 particles. Forty eight hours later, acetaminophen was delivered i.p. as a single bolus of 300 mg/kg, a

dose identified to fail to give rise to any significant toxicity in earlier experiments (data not shown) and was consistent with values reported in the literature.

Tumor growth was monitored in those mice that received the combination of Ad.mCYP1A2 and acetaminophen, and compared to control groups infected with Ad.mCYP1A2 plus prodrug vehicle and those mice that were injected with virus vehicle only and exposed to acetaminophen. A substantial anti-tumour effect was evident in those xenografts that received Ad.mCYP1A2 and acetaminophen, indicated by the reduced average tumour volume (Fig. 6A). Additionally, overall animal survival was significantly increased compared to the control groups, 4 out of 5 mice (80%) were still alive after 100 days of follow up observations compared to 20% (virus vehicle + acetaminophen) and 0% (Ad.mCYP1A2 + prodrug vehicle) for the control groups (Fig. 6B). Similar results were obtained in two additional independent HepG2 experiments (data not shown).

The P450 Enzyme/Acetaminophen GDEPT Approach of the Present Invention Is Highly Likely to Succeed Clinically

27. I understand that for a successful GDEPT approach to cancer treatment, effective gene delivery vehicles and expression technology, the nature of the partner prodrug are important, and concerns of undesirable toxicities and poorly understood pharmacokinetics and pharmacodynamics should be addressed. However, the use of acetaminophen (paracetamol) for a P450 enzyme-based system is very promising as acetaminophen, at therapeutic doses, is a safe and well characterized drug in the human population and several drug strategies are available to reduce/prevent toxicity mainly located to the liver following an overdose , and thereby to increase the therapeutic index.

In this regard, it is notable that mice are more sensitive, mass for mass, to acetaminophen-mediated hepatotoxicity than are humans (see toxicity data published by National Institute for Occupational Safety and Health online at www.cdc.gov/niosh/rtecs/ae401640.html

28. Furthermore, several properties make it an attractive system: CYP1A2/acetaminophen killing is independent of cell proliferation, independent of p53 status, and the bystander effect is achieved by the membrane diffusible activated drug NABQI.

29. More importantly, the data above showed that acetaminophen in combination with clinically relevant adenoviral vectors has been demonstrated to be effective *in vivo*.

29. It is therefore my opinion that a skilled person engaged in gene-therapy would expect that the CYP1A2/acetaminophen approach described in the instant application would highly likely be successful in humans.

30. I further declare that all statements made herein of my own knowledge are true , and all statements made on information or belief are believed to be true, and further that there statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity or enforceability of the above-identified United States patent application or any patent issued thereon.

Signed this 21 day of January, 2005

Donald Davies

#348224

Table 1

Cell line	Tumor type	Relative susceptibility
Hep G2	HCC	++
HCT116	CaColon	++
SW480	CaColon	++
DU145	CaProstate	+
Hepa 1-6*	HCC	+++
CMT93*	CaColon	+++
B16*	Melanoma	+
4T1*	CaBreast	+++

* Murine tumors

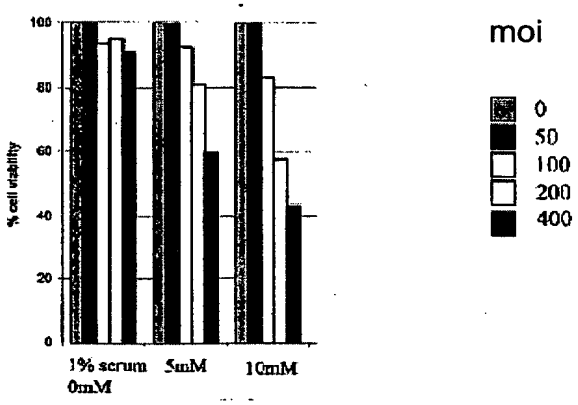

Figure 1. CTL706 (hCYP1A2) infection of HepG2 cells WITHOUT BSO. HepG2 cells are sensitized to acetaminophen by Ad-mediated expression of human CYP1A2. HepG2 cells were infected with the indicated MOIs of Ad.hCYP1A2 and the indicated acetaminophen concentrations. hCYP1A2 expression was allowed for 24 h, cells were then incubated overnight with prodrug and cell viability was then evaluated 48 h later with MTS substrate assay kit. One representative experiment and the SD is shown.

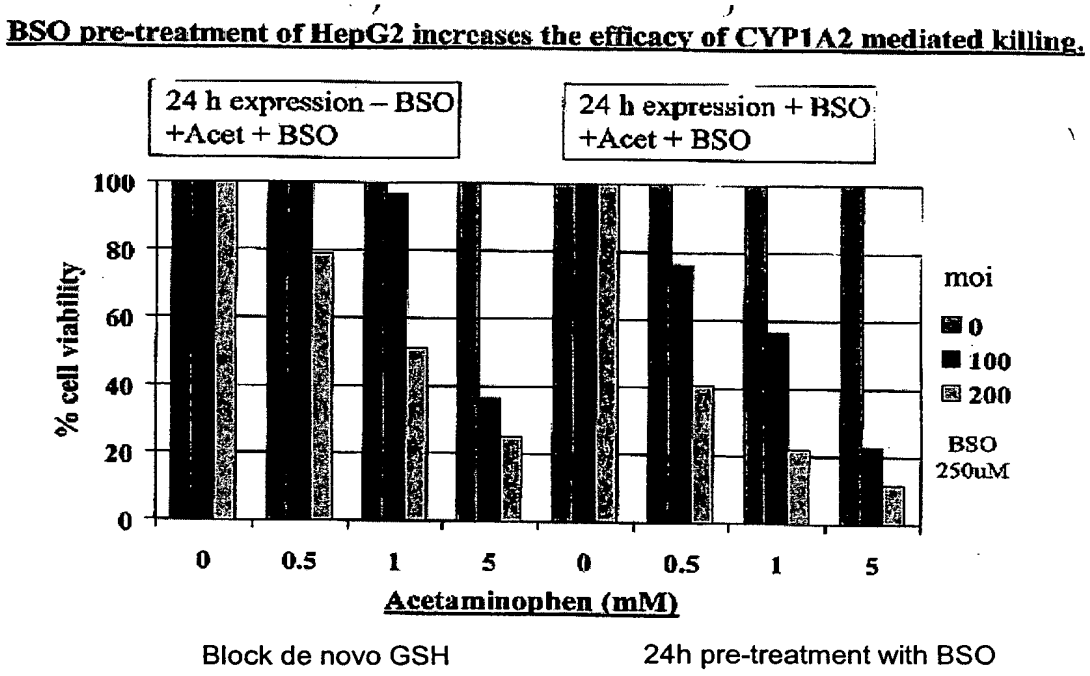

Figure 2. Blocking GSH levels by BSO improves hCYP1A2/acetaminophen cell killing. HepG2 cells were infected with the indicated MOIs of Ad.hCYP1A2 in the absence (left) or presence of BSO. After 24h allowing transgene expression and depletion of GSH by BSO, cells were given acetaminophen at the indicated concentrations including 250 μM BSO. Cell viability was then evaluated 48 h later with MTS substrate assay kit. One representative experiment and the SD is shown.

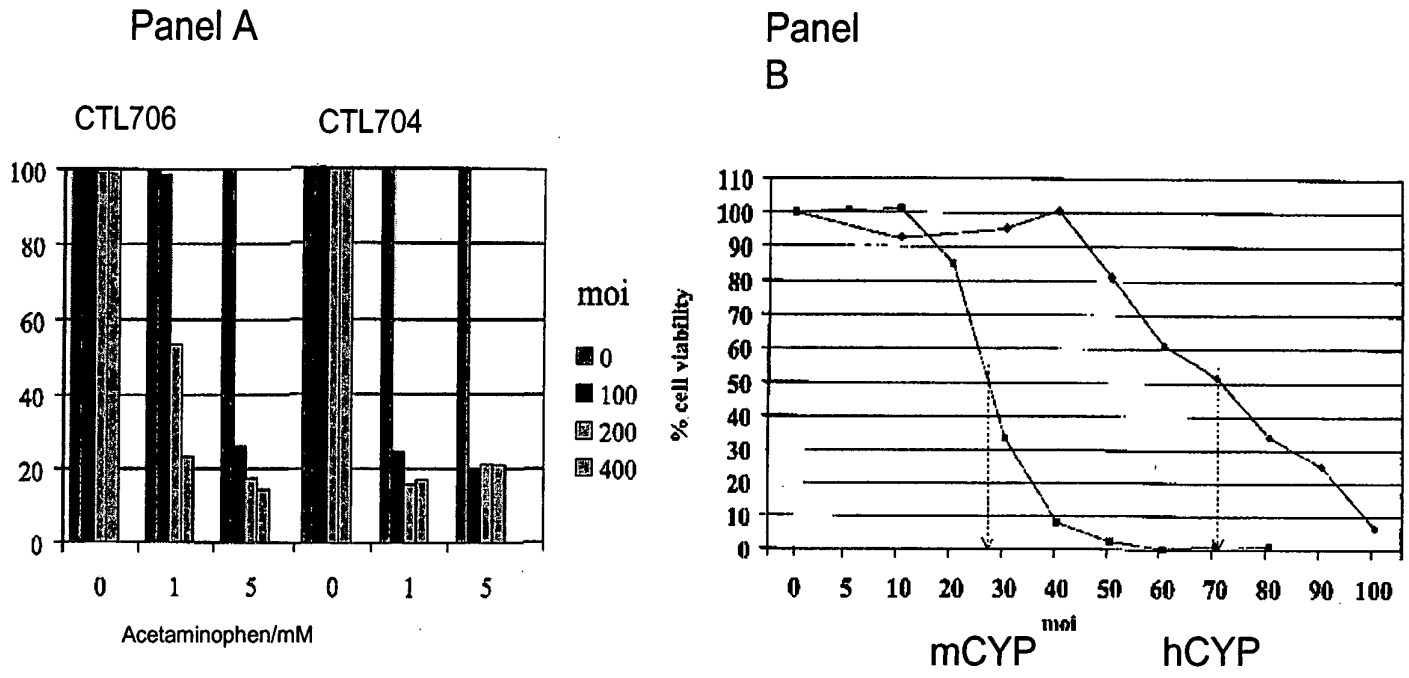

Figure 3. mCYP is more active than hCYP. Mouse CYP1A2 shows increased killing properties compared to human CYP1A2. (A) HepG2 cells were infected at the indicated MOIs of either Ad.hCYP1A2 or Ad.mCYP1A2 and 24 h later cells were treated with increasing acetaminophen concentrations and in the presence of 250 μM BSO. (B) Cells were infected with either Ad.hCYP1A2 or Ad.mCYP1A2 across a broad range of MOIs and 24 h later cells were treated 5 mM acetaminophen in the presence of 250 μM BSO. Shown is the dose/response curve for both viruses and indicated is the MOI for each virus needed to achieve reduction to 50% cell viability. Cell viability was determined using the MTS substrate assay kit. One representative experiment is shown.

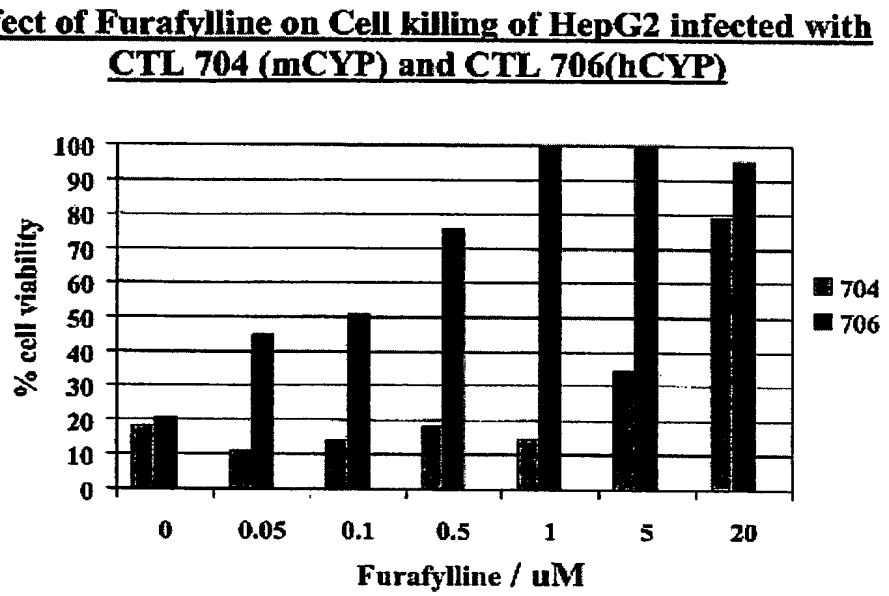

Figure 4. Human CYP1A2 is selectively inhibited by Furafylline. HepG2 cells were infected with either Ad.hCYP1A2 or Ad.mCYP1A2 at an MOI of 200. After 24 h cells were incubated with Furafylline (0-20 μM) for 60 min, followed by the addition of 5 mM acetaminophen in the presence of 250 μM BSO. About 24 h later cell viability was then evaluated with MTS substrate assay kit. One representative experiment is shown.

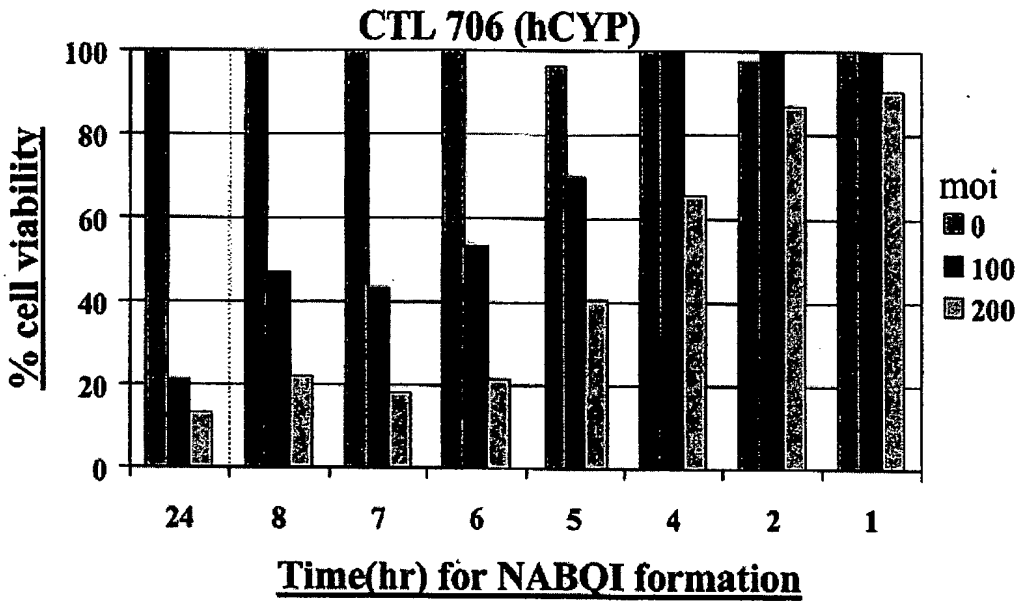

Figure 5. Six hours exposure to acetaminophen is sufficient to kill HepG2. HepG2 cells were infected with the indicated MOIs of Ad.hCYP1A2 and enzyme expression was allowed for 24 h. Cells were then incubated in 5 mM acetaminophen from 1 to 24 h prior to the addition of 2 μM Furafylline to inhibit any further hCYP1A2 activity. Cell viability was analysed 24 h later with the MTS substrate at staggered time points to ensure that the time for cell viability determination following prodrug exposure was constant. One representative experiment is shown.

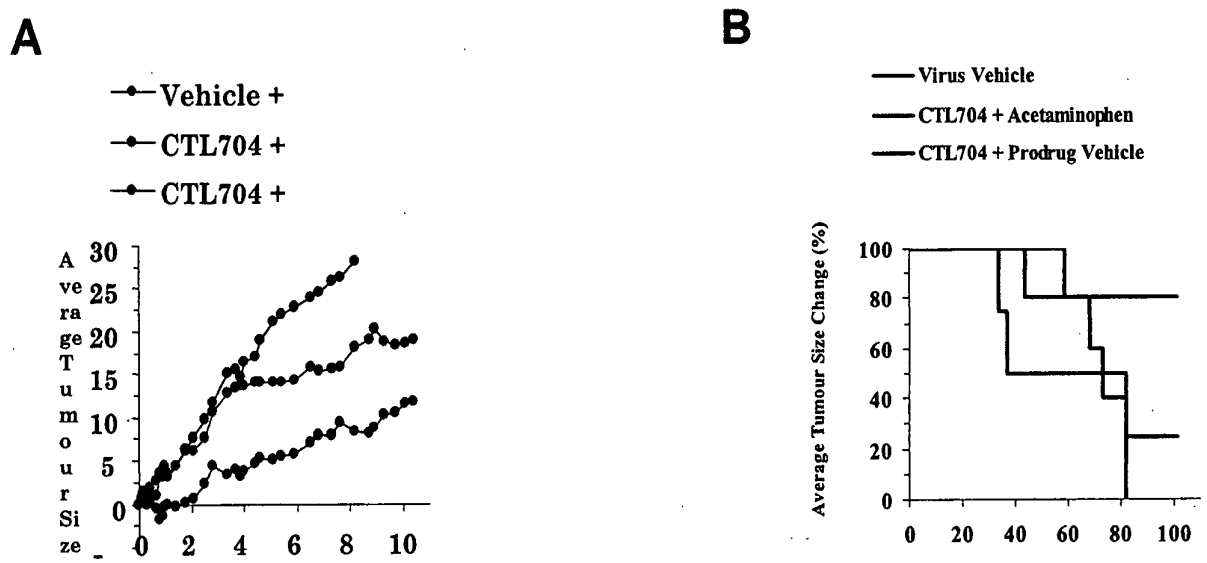

Figure 6. Ad.mCYP1A2/acetaminophen shows in vivo efficiency against HepG2 xenografts. HepG2 tumour xenografts were engrafted on Balb/C mice and allowed to grow to palpable size. Tumours were then directly injected with 1.0 x 10^{10} particles of Ad.mCYP1A2. After 48 h acetaminophen was delivered i.p. as a single bolus of 300 mg/kg. Control groups included virus vehicle/acetaminophen and Ad.mCYP1A2/ prodrug vehicle. Each group contained 5 animals. Shown is the average tumour size of each group (A) and percentage of animal survival (B). One out of three representative HepG2 experiments is shown.